REMARKS

Claims 1-9, 11, and 16-51 have been canceled. Claims 10 has been amended. New Claims 52-62 have been added. Support for these claim amendments can be found throughout the specification and the originally filed claims. For example, on page 3, lines 14-17, page 4, lines 1-4, page 7, lines 7-12, page 18, line 21 to page 19, line 4. No new matter has been added. As such, Claims 10, 12-15, and 52-62 will be pending upon entry of this amendment.

35 U.S.C. § 112 REJECTION

Claims 1, 3-7, 10, 12-15, 18, 20-25, 28 and 30-35 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly not enabling one skilled in the art to make and use the invention. As noted above, Claims 1, 3-7, 18, 20-25, 28, and 30-35 have been canceled. Applicants thus address this rejection with respect to pending claims 10 and 12-15.

The presently claimed invention is directed to methods of treating and/or preventing a cardiovascular disease characterized by platelet activating factor activity and/or superoxide generation in a human in need of such treating and/or preventing which is not suffering from an allergic and/or inflammatory condition of the skin or upper airway passages which comprises administering to said human an effective amount of desloratadine, or a pharmaceutically acceptable salt thereof, to reduce the risk or prevent the occurrence of said cardiovascular disease.

The specification discloses that desloratadine attenuated platelet activating factor activity as well as spontaneous superoxide generation in humans. See the specification, for example, on page 18, line 12 to page 19, line 4. The results presented in the specification apply to cardiovascular disease characterized by platelet activating factor activity and/or superoxide generation for which the articles submitted herein as Appendix A and Appendix B below provide additional support.

The nexus between platelet activating factor activity and cardiovascular disease is supported by Hospers *et al.*, "Eosinophilia and positive skin tests predict cardiovascular

mortality in a general population sample followed for 30 years," *Am J Epidemiol*, 150(5):482-491 (1999), a copy of which is submitted herewith as Appendix A. Clearly, cardiovascular mortality does not occur in the absence of cardiovascular disease.

Likewise, the nexus between superoxide generation and cardiovascular disease is supported by the review article Li and Shah, "Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology," *Am J Physiol Regul Integr Comp Physiol*, 287:R1014-R1030 (2004), a copy of which is submitted herewith as Appendix B.

Note that the article previously cited by the Examiner, "NIH Heart Disease & Stroke Research: Fact Sheet" (American Heart Association, 2004); "Cardiovascular Disease: Treatment for Stroke," Stanford Hospital & Clinics, 2003, distinguishes prevention, treatment and cure of heart disease as all 3 terms are recited on page 1, last paragraph. This article also recognizes current research regarding treatment and prevention strategies for reducing the death rate from cardiovascular disease. See page 2 of 3, 4th paragraph. The presently claimed invention is directed to methods of treating and/or preventing a cardiovascular disease (characterized by platelet activating factor activity and/or superoxide generation) using desloratadine or a pharmaceutically acceptable salt thereof, to reduce the risk or prevent the occurrence of such a cardiovascular disease. See, the specification, for example, at page 3, lines 14-17. A reduction in the risk of cardiovascular disease can be ascertained as described in the specification, for example, on page 15, lines 15-21.

In light of the claim amendments, article submissions, and comments detailed above, Applicants' respectfully request withdrawal of the aforementioned 35 U.S.C. § 112, first paragraph rejection of Claims 10 and 12-15.

35 U.S.C. § 102 Rejections

Claims 1, 3-7, 10, 12-15, 18, 20-25, 28, 30-33 and 35 are rejected under 35 U.S.C. §102(b) as being anticipated by Aberg et al. (U.S. 5,731,319). As noted above, Claims 1,

3-7, 18, 20-25, 28, and 30-35 have been canceled. Applicants thus address this rejection with respect to pending claims 10 and 12-15.

Notably, the presently claimed invention is directed to a different treatment group than Aberg *et al.* Namely, in contrast to Aberg *et al.*, the treatment group of the presently claimed invention is humans which are not suffering from an allergic and/or inflammatory condition of the skin or upper airway passages. Rather, the treatment group of the presently claimed invention is humans which are in need of treating and/or preventing a cardiovascular disease characterized by platelet activating factor activity and/or superoxide generation. As such, Aberg *et al.* can not possibly anticipate the presently claimed invention either expressly or inherently.

In light of the claim amendments and comments detailed above, Applicants' respectfully request withdrawal of the aforementioned 35 U.S.C. § 102 rejection of Claims 10 and 12-15.

35 U.S.C. § 103 Rejections

Claims 1, 3-7, 10, 12-15, 18, 20-25, 28 and 30-35 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Aberg *et al.* (U.S. 5,731,319) in view of Buckland *et al.* (EP 0 968 715 A1), and further in view of Gray (US 5,627,183), Kreutner *et al.* (US 5,869,479) and Hospes *et al.*, (*J Epidemiol*, **150(5)**:482-491 (1999)). As noted above, Claims 1, 3-7, 18, 20-25, 28, and 30-35 have been canceled. Applicants thus address this rejection with respect to pending claims 10 and 12-15.

As noted above, in contrast to Aberg *et al.*, the treatment group of the presently claimed invention is humans which are not suffering from an allergic and/or inflammatory condition of the skin or upper airway passages. Not only do Aberg *et al.* disclose a different treatment group, but also a different indication. Aberg *et al.* is directed to methods for treatment of allergic rhinitis and other disorders (namely, allergic asthma, cough, cold, cold-like, and/or flu symptoms and the discomfort, headache, pain, fever, and general malaise associated therewith, retinopathy or other small vessel diseases

associated with diabetes mellitus) using desloratadine while avoiding the concomitant liability of adverse side-effects associated with other non-sedating antihistamines. See, Aberg et al., column 4, line 65 to column 5, line 62. Notably, such adverse side-effects include, but are not limited to, tumor promotion, cardiac arrhythmias, cardiac conduction disturbances, fatigue, headache, gastrointestinal distress, appetite stimulation, weight gain, dry mouth, constipation or diarrhea. See, Aberg *et al.*, column 3, lines 27-31 and lines 42-45. Aberg *et al.*, however, provides no guidance for treating and/or preventing cardiovascular disease, particularly those characterized by platelet activating factor activity and/or superoxide generation.

In fact, not only does Aberg *et al.* fail to disclose or suggest the use of desloratadine to treat or prevent cardiovascular disease, but Aberg *et al.* actually teach away from this indication as Aberg *et al.* disclose that loratadine or desloratadine may be administered without side effects such as cardiac arrhythmias or cardiac conduction disturbances. Far from disclosing the methods of the instant invention, then, Aberg *et al.* reportedly discovered methods which teach away from the administration of desloratadine to treat and/or prevent cardiovascular disease because Aberg *et al.* report that the disclosed methods would not have cardiovascular effects – either positive or negative. The fact that Aberg *et al.* contains negative teachings which would discourage and deter a person of ordinary skill in the art from using desloratadine for the treatment and prevention of cardiovascular disease is evidence of non-obviousness.

Buckland *et al.* is directed to the use of loratadine as an antiarrhythmic. Buckland *et al.* notes that according to the prior art "loratadine, although having properties which may prevent atrial arrhythmia, may also have the potential for initiating this arrhythmia." See page 2, lines 24-31. However, Buckland *et al.* does not resolve this disparity in the prior art as it does not present any data to resolve this conflict but merely discloses an assay. Thus, one of skill in the art faced with this discrepancy in the prior art would not be motivated to use loratadine as an antiarrhythmic.

Furthermore, assuming arguendo that Buckland *et al.* uses desloratadine to treat cardiovascular disease such as arrhythmia, no line of reasoning was presented as to why a skilled artisan reviewing only the combined teachings of the references would have found it obvious from the references relied upon to arrive at the claimed invention particularly as Aberg *et al.* teaches away from the use of desloratadine for the treatment and prevention of cardiovascular disease.

Likewise, adding either the Gray, Kreutner or Hospes references does not remedy the defects of Aberg *et al.* or Buckland *et al.*

Kreutner allegedly discloses that desloratadine is a functional equivalent of cetirizine. However, Kreutner also discloses a number of other antihistamines. Kreutner specifically states that one could employ "astemizole, azatadine, azelastine, acrivastine, brompheniramine, chlorpheniramine, clemastine, cyclizine, carebastine, cyproheptadine, carbinoxamine, descarboethoxyloratadine (also known as SCH-34117), doxylamine, dimethindene, ebastine, epinastine, efletirizine, fexofenadine, hydroxyzine, ketotifen, loratadine, levocabastine, mizolastine, mequitazine, mianserin, noberastine, meclizine, norastemizole, picumast, pyrilamine, promethazine, terfenadine, tripelennamine, temelastine, trimeprazine and triprolidine" (col. 2, li. 52-62) in the disclosed method.

The Office has not indicated why one of skill in the art would choose desloratadine as opposed to any one of the other antihistamines disclosed in Kreutner to treat and/or prevent cardiovascular disease. The Office has not pointed to any evidence as to why one of ordinary skill in the art would be motivated to select desloratadine to the exclusion of the other compounds on that list and combine it with Aberg *et al.* or any other reference to achieve the claimed invention.

Moreover, one skilled in the art would not be motivated to substitute desloratadine for cetirizine because the compounds have different indications. Further, desloratadine is not sedating while cetirizine is. Further, the two compounds have significantly different

chemical structures. Therefore, a person of ordinary skill in the art would not readily interchange one compound for the other.

Even assuming, however, that one of ordinary skill in the art would know to substitute desloratadine for cetirizine, like Aberg *et al.*, both Gray and Kreutner teach away from the claimed invention. Specifically, Gray provides that the claimed invention avoids certain side effects, including "sedation and somnolence, headache, gastrointestinal disturbance, dizziness, nausea, cardiac arrhythmias and other cardiovascular effects" (col. 3, li. 61-62). Similarly, Kreutner recites, "The decongestant effect of the combination of the present invention is thought to reside in the anti-H_3 activity which enhances the release of norepinephrine, a natural endogenous decongestant, at the site of congestion in the nose, but not elsewhere in the body, so no systemic cardiovascular effects are observed" (col. 3, li. 56-62). Aberg *et al.*, Gray and Kreutner all teach away from the use of desloratadine for treatment and/or prevention of cardiovascular disease because the teachings suggest that the disclosed methods have no cardiac effects – positive or negative. The combination of Aberg *et al.* with Gray or Kreutner does not suggest or teach the claimed invention, even with the teaching of the other references, the combination still also does not result in the use of desloratadine to treat and/or prevent cardiovascular disease as is recited in claims 10 and 12-15.

Moreover, with respect to Hospes, it is respectfully submitted that applicants have not, in fact, admitted that the reference is prior art. Mere citation to a reference does not transform it into prior art; nor does characterization of a reference as prior art by the Office transform that reference into prior art.

Hospes also fails to disclose or suggest the use of desloratadine to prevent or treat cardiovascular disease. Therefore, Aberg *et al.* and Hospes in combination do not teach or suggest the claimed invention, and, even with Buckland *et al.* or any of the other references, the combination of references does not result in the use of desloratadine to prevent or treat cardiovascular disease.

In light of the claim amendments and comments detailed above, Applicants' respectfully request withdrawal of the aforementioned 35 U.S.C. § 103 rejection of Claims 10 and 12-15.

Double Patenting Rejection

Claims 1, 3-7, 10, 12-15, 18, 20-25, 28, 30-35 stand rejected under the judicially created doctrine of double patenting over claims 1-4 of U.S. Patent No. 6,114,346 ("'346 patent") or claims 1-3 of U.S. Patent No. 6,265,414 ("'414 patent") and claims 1-13 of U.S. Patent No. 6,432,972 ("'972 patent"). As noted above, Claims 1, 3-7, 18, 20-25, 28, and 30-35 have been canceled. Applicants thus address this rejection with respect to pending claims 10 and 12-15.

As the presently claimed invention is directed to a different treatment group than those disclosed in the aforementioned patents, the double patenting rejection no longer applies. Namely, the treatment group of the presently claimed invention is humans which are not suffering from an allergic and/or inflammatory condition of the skin or upper airway passages. In addition, , the treatment group of the presently claimed invention is humans which are in need of treating and/or preventing a cardiovascular disease characterized by platelet activating factor activity and/or superoxide generation.

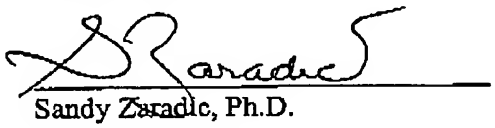
The '346 and the '414 patents claim methods of treating and preventing sleep disorders in humans with upper airway passage allergies using desloratadine. The '972 patent claims methods of treating and/or preventing congestion using desloratadine. Unlike the presently claimed invention, none of these patents claim a method of treating and/or preventing cardiovascular disease using desloratadine.

In light of the above claim amendments and comments, Claims 10 and 12-15 are believed to be overcome this double patenting rejection.

CONCLUSION

It is believed that the foregoing amendments and arguments place this application now in condition for allowance. Therefore, favorable action allowing pending Claims 10, 12-15, and 52-62 is respectfully solicited.

Respectfully submitted,



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APPENDIX A

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Eosinophilia and Positive Skin Tests Predict Cardiovascular Mortality in a General Population Sample Followed for 30 Years

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The authors investigated whether two objective allergy markers, peripheral blood eosinophilia and skin tests for common aeroallergens, were associated with cardiovascular death. Of 5,382 subjects in the Vlagtwedde-Vlaardingen Study (the Netherlands) with data on allergy markers in 1965–1972, 507 subjects died from cardiovascular disease during 30 years of follow-up. Subjects with eosinophilia had an increased risk of cardiovascular death (relative risk (RR) = 1.7; 95% confidence interval (CI): 1.4, 2.2), including ischemic heart disease death (RR = 1.6; 95% CI: 1.2, 2.2) and cerebrovascular death (RR = 2.3; 95% CI: 1.4, 3.8), independent of major risk factors. This association was limited to subjects with a percentage of the predicted forced expiratory volume in 1 second (FEV_1 % predicted) of <100%. Positive skin tests were associated with a significantly reduced cardiovascular mortality in subjects with normal lung function and weight who did not smoke (RR = 0.15; 95% CI: 0.05, 0.46). Conversely, when subjects with positive skin tests had a body mass index of ≥25 kg/m², had an FEV_1 % predicted of <80%, or smoked, they had an increased risk for cardiovascular mortality. These results were not restricted to asthmatics. Our data suggest a possible link between eosinophilia and positive skin tests and cardiovascular mortality, especially in combination with other risk factors associated with its mortality. *Am J Epidemiol* 1999;150:482–91.

allergens; cardiovascular diseases; cohort studies; eosinophilia; eosinophils; mortality; population; skin tests

Smoking, hypertension, and hypercholesterolemia are established risk factors for cardiovascular disease (1–4). Other factors including physical inactivity (1), diabetes mellitus (1), high body mass index (3), and low percentage of the predicted forced expiratory volume in 1 second (FEV_1) (5, 6) are also associated with increased risk of cardiovascular disease. However, other yet unrecognized factors must play a role, since these risk factors do not explain all prevalence of cardiovascular disease (2, 4).

Allergy might be such a factor (7). Type I allergy or atopy occurs frequently in the population and has been reported to be increasing in Western countries (8). Therefore, an association between allergy and cardiovascular disease is potentially important. Allergy can be studied subjectively by using questionnaires or objectively by using biologic markers, i.e., peripheral blood eosinophil counts, total and specific serum immunoglobulin E levels, and skin tests. Prentice et al. (9) showed that the eosinophil fraction of the total leukocyte count was associated with increased coronary heart disease incidence after adjustment for total leukocyte count but not for smoking habits. They further showed that at a specified leukocyte count the estimated coronary heart disease risk increased as the eosinophil fraction relative to the neutrophil fraction increased. Recently, Sweetnam et al. (10) reported that, besides total leukocyte count and neutrophil count, an elevated peripheral blood eosinophil count also was associated with an increased incidence of ischemic heart disease. They did not adjust for total leukocyte count or neutrophil count (10), but their results were adjusted for smoking, a well-known risk factor for cardiovascular disease (11). In contrast with the former two studies, Olivares et al. (12) found no association between the leukocyte subpopulations eosinophilia and neutrophilia, analyzed as absolute and proportional counts, and incidence of ischemic heart disease (only 46 cases) after adjustment for age and smoking. It is, therefore, a controversial issue whether smoking influences the association between eosinophilia and the incidence of ischemic heart disease.

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Abbreviations: CI, confidence interval; FEV_1, forced expiratory volume in 1 second; ICD, *International Classification of Diseases*; RR, relative risk.

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A similar argument holds for reduced levels of lung function. Low FEV_1 is associated with a higher peripheral blood eosinophil count (13, 14) and with a higher incidence of cardiovascular disease, independent of smoking (5, 6, 15, 16). On the basis of clinical observations, Rozencwaig (7) suggested a higher incidence of cardiovascular disease in patients with positive allergy tests, but the fact that he did not adjust for other risk factors for cardiovascular disease may have confounded the association between allergy and the incidence of cardiovascular disease. Cardiovascular disease is the most important cause of death in Western countries. Smoking and FEV_1 are not only associated with cardiovascular disease but also with cardiovascular mortality (6, 17).

We were interested whether subjects with objective allergy markers, peripheral blood eosinophilia and/or positive skin tests to common aeroallergens, have increased cardiovascular mortality from ischemic and cardiovascular diseases other than ischemic heart disease, after adjustment for major risk factors. We also investigated whether these associations were restricted to asthmatic individuals, since allergy exists in almost all patients with asthma (18).

MATERIALS AND METHODS

Population

The Vlagtwedde-Vlaardingen Study is an epidemiologic field study of risk factors for asthma and chronic obstructive pulmonary disease in a random sample of the inhabitants of two Dutch communities. The population selection has been described previously (19, 20). Briefly, in 1965 this study was carried out in Vlagtwedde, a rural area in the northeast, and in Vlaardingen, an urban area in the western part of the Netherlands, in subjects aged 40–64 years, followed by a survey of young people aged 15–39 years in 1967 in Vlagtwedde and in 1969 in Vlaardingen. In some cases, eosinophil counts were not available in the first survey but were available in the first follow-up survey in 1970 in Vlagtwedde or in 1972 in Vlaardingen, and in those cases data from the first follow-up were used (629 cases). A total of 6,378 subjects had a peripheral blood eosinophil count available, and 6,324 had both a peripheral blood eosinophil count and skin tests available; 117 subjects were excluded because of missing data on lung function, smoking, weight, or asthma status. Because of the unsatisfactory quality (n = 795) or lack of an assessment (n = 30) of the spirogram, another 825 subjects were excluded. A total of 5,382 men and women had peripheral blood eosinophil counts, skin tests, and complete data for all other covariates.

Peripheral blood eosinophil count

Peripheral blood eosinophil counts were assessed in a 1:11 dilution of peripheral blood using a Bürker counting chamber (18, 20). Eosinophilia was defined as ≥275 cells/mm³ of blood, a cutoff point based on the investigation by Veening (21).

Allergen skin testing

Four common aeroallergens were applied intracutaneously to the volar forearm: house dust, mixed pollen, epidermal products, and mixed molds (20, 22). Wheal diameters for each allergen were measured to the nearest 0.5 mm and coded on a six-point scale (0 = 0–5 mm, 1 = 5.1–7.5 mm, 2 = 7.6–10 mm, 3 = 10.1–12.5 mm, 4 = 12.6–15 mm, 5 = >15 mm). Scores for the four allergens were added to a skin test sum score (range, 0–20). Positive skin tests were defined as a skin test sum score of ≥3 (18). A histamine biphosphate solution was used as a positive control (20, 22). Eight subjects had no data on the positive control, and 391 subjects had no reaction to the positive control.

Body mass index

Body mass index was calculated as weight (kg)/height (m)² and divided into four classes according to World Health Organization criteria: underweight, <18.50; normal weight, 18.50–24.99; overweight, 25.00–29.99; and obesity ≥30 kg/m² (23).

Questionnaire

Data on age, sex, smoking habits, and respiratory symptoms were collected by means of a Dutch version of the British Medical Research Council's standard questionnaire (24–26). Interviews were performed by trained interviewers. Smoking was considered as a categorical variable; exsmokers had stopped smoking at least 1 month before the examination, and current smokers smoked ≥1 cigarette a day. Pipe and cigar smokers were also considered as smokers. Asthma was considered to be present if an affirmative answer was given to the question of whether a subject had ever experienced attacks of shortness of breath with wheezing at rest (asthma attacks). Thus, asthma was self-reported and not physician-diagnosed asthma.

Spirometry

FEV_1 was assessed with a water-sealed spirometer (D 53; Lode Instruments, Groningen, the

Netherlands). Measurement of inspiratory vital capacity after a deep expiration was followed by the measurement of FEV_1. Subjects performed the maneuver until two technically satisfactory tracings were produced, the higher value of these tracings being taken as the baseline measurement (13, 20). The percent predicted reference values were calculated with regression coefficients derived from analysis of our own population data. For all asymptomatic subjects regardless of their smoking habits who took part in the 1965/1967/1969 survey 1 or, when no complete data for all covariates were available from this first survey, in the 1970/1972 survey 2, we computed regression equations for FEV_1 as a function of age and height, with an age cutoff of 21 years for both sexes separately. The actual measured FEV_1 value was expressed as the percentage of the predicted FEV_1. FEV_1 was used in the analyses as <80 percent of predicted for subjects with reduced and as ≥80 percent of predicted for subjects with (near) normal lung function, as reported by Lange et al. (6). The latter group was divided into subgroups with near normal (80–99.99 percent) and normal (≥100 percent of the FEV_1 percent predicted) lung function.

Follow-up

Until March 10, 1995, subjects were traced for their vital status (alive, dead, lost to follow-up) with 99 percent success (4,172 alive and 1,135 dead); only 75 subjects (1 percent) were lost to follow-up. Survival time was calculated for each subject from the date of entry into the study (between 1965 and 1972) until the end of follow-up at one of the following three times: 1) March 10, 1995, for subjects registered at the municipalities as being alive; 2) the date of death for subjects identified in the Death Register of the municipalities; or 3) the last registration of subjects lost to follow-up, for example, date of last survey attended or date of move when the new address could not be traced. Causes of death were obtained from the Netherlands Central Bureau of Statistics in Voorburg. Cardiovascular death was classified according to *International Classification of Diseases* (ICD) codes for cardiovascular disease during 1965–1995: ICD, Revisions 7, 8, and 9 (ICD-7, ICD-8, and ICD-9, respectively). Cardiovascular death was divided into that due to ischemic and cardiovascular diseases other than ischemic heart disease, with the latter group subdivided into death due to cerebrovascular and other cardiovascular diseases. This last subgroup included death due to rheumatic heart disease, diseases of the arteries, hypertensive disease, other heart disease, other diseases of the vascular system, pulmonary embolism, and (sub)acute endocarditis according to the classification of Mackenbach et al. (27, 28) (see Appendix).

Statistical analyses

Associations between the allergy markers and mortality from cardiovascular disease were estimated with the Cox proportional hazard model (29). Time was defined from the initial examination until the endpoint of interest, either cardiovascular death or one of its subgroups. Censoring took place when the subjects were still alive at March 10, 1995, were lost to follow-up (only 1 percent), or died from another cause (30). A proportional hazards model accounts for varying intervals in follow-up among subjects (e.g., censoring) and permits for control the potential confounding effects of other risk factors. Analyses were performed with simultaneous adjustment for city (Vlaardingen vs. Vlagtwedde), sex, age, smoking habits, FEV_1 percent predicted, and body mass index at the start of the study (table 3). Analyses were repeated for the two subgroups of cardiovascular death, ischemic and cardiovascular diseases other than ischemic heart disease, and for the two subgroups of death due to cardiovascular diseases other than ischemic heart disease, cerebrovascular death and death due to other cardiovascular disease. Differences among subgroups of sex, age, smoking habits, lung function, body mass index, and city were tested by inclusion of an interaction term with the allergy markers in the model. In addition, we added a history of asthma attacks to this model, as both a single term and a term for interaction with the allergy markers, to test whether subjects with both allergy and asthma had a higher risk of mortality from cardiovascular disease. If an interaction with one of these covariables was statistically significant (p value < 0.05), it was added to the model (table 4).

RESULTS

Table 1 shows the population characteristics at the start of the study stratified by the absence or presence of eosinophilia and positive skin tests. Twelve percent (662 subjects) had eosinophilia, and 15 percent (834 subjects) had positive skin tests. Only 3 percent (161 subjects) had both eosinophilia and positive skin tests. Subjects with eosinophilia were more often male, smoked more often, had lower levels of FEV_1 percent predicted, had more often a history of asthma attacks, and lived more often in Vlaardingen than did subjects without eosinophilia. Subjects with positive skin tests were more often male, smoked more often, were younger, had a lower body mass index, had more often a history of asthma attacks, and lived more often in Vlaardingen than did subjects without positive skin tests.

TABLE 1. Baseline characteristics of 5,382 subjects at the start of the study in 1965–1972 for all subjects, subjects without and with eosinophilia or positive skin tests, Vlagtwedde-Vlaardingen Study, the Netherlands

	Total subjects		Men (%)	Mean age (years)	Smoking (%)			Mean FEV_1* % predicted	FEV_1 % predicted (%)		
	No.	%			Never	Exsmoker	Yes		≥100%	80–99.99%	<80%
Total	5,382	100	54	36 (13)†	36	9	55	98 (15)	47	44	9
Eosinophilia											
Subjects without	4,720	88	54	36 (13)	36	9	55	99 (15)	48	44	8
Subjects with	662	12	58	35 (14)	32	8	60	95 (17)	39	46	15
p value‡			0.03	NS*		0.02		<0.001			
Positive skin tests											
Subjects without	4,548	84	53	37 (13)	36	9	55	98 (15)	47	44	9
Subjects with	834	15	60	31 (12)	32	9	59	96 (15)	48	43	9
p value			<0.001	<0.001		0.01		NS			

	% with the following body mass index (kg/m²)				Asthma		Subjects with positive skin tests		Subjects with eosinophilia		Residence (%)	
	<18.50	18.50–24.99	25.00–29.99	≥30.00	No.	%	No.	%	No.	%	Vlaardingen	Vlagtwedde
Total	2	50	38	10	162	3	834	16	682	12	45	55
Eosinophilia												
Subjects without	2	50	38	10	128	3	673	14			43	57
Subjects with	2	54	35	9	54	8	161	24			59	41
p value		NS			<0.001		<0.001				<0.001	
Positive skin tests												
Subjects without	2	49	39	10	119	3			501	11	43	57
Subjects with	2	59	33	6	63	8			161	19	55	45
p value		<0.01			<0.001				<0.001		<0.001	

* FEV_1, forced expiratory volume in 1 second; NS, not significant at $p < 0.05$.
† Numbers in parentheses, standard deviation.
‡ *p* values calculated by chi-square or *t* test.

Table 2 shows that, of the 1,135 deaths occurring during follow-up, 507 (45 percent) were due to cardiovascular disease: 89 (51 percent) in the group with eosinophilia and 44 (43 percent) in the group with positive skin tests. There were 303 deaths (27 percent) from ischemic heart disease, 51 (29 percent) in the group with eosinophilia and 25 (25 percent) in the group with positive skin tests. There were 204 deaths (18 percent) from cardiovascular diseases other than ischemic heart disease (nonischemic heart death in the table), of which 95 (8 percent) died from cerebrovascular disease and 109 (10 percent) subjects died from other cardiovascular disease.

Eosinophilia was associated with an increased risk for death from cardiovascular disease (relative risk (RR) = 1.73; 95 percent confidence interval (CI): 1.37, 2.18), independent of skin tests, sex, age, smoking habits, FEV_1 percent predicted, body mass index, and city at the start of the study (table 3). This increased mortality risk was present for both ischemic heart disease (RR = 1.64; 95 percent CI: 1.21, 2.23) and cardiovascular diseases other than ischemic heart disease (RR = 1.92; 95 percent CI: 1.34, 2.74). In the latter group, death from cerebrovascular disease (RR = 2.30; 95 percent CI: 1.39, 3.79) was significantly increased. In contrast, the presence of positive skin tests was not associated with increased mortality from all cardiovascular diseases (RR = 1.03; 95 percent CI: 0.75, 1.42) or any of the subgroups. The association between eosinophilia and cardiovascular mortality was not different for subjects with and without positive skin tests. Furthermore, sex, age, smoking, FEV_1 percent predicted, and body mass index were all associated with increased mortality from cardiovascular disease and ischemic heart disease, but not the variables exsmoking and city. Smoking and overweight were not associated with death due to cardiovascular diseases other than ischemic heart disease (nonischemic deaths in table 3) and its subgroups. In contrast, underweight (body mass index of <18.50 kg/m²) was associated with death due to cardiovascular diseases other than ischemic heart disease and its subgroups and not with ischemic heart death. Finally, subjects in Vlaardingen had a significantly increased risk for death from cardiovascular diseases other than ischemic heart disease.

When the presence of self-reported asthma and the interaction terms of asthma with the allergy markers were added to this model, the associations of eosinophilia and increased risk of cardiovascular death and its subgroups remained unchanged (table 3, lower

TABLE 2. Follow-up data and specification of cardiovascular death causes during 30 years of follow-up for all subjects who participated in the Vlagtwedde-Vlaardingen Study (the Netherlands) in 1965–1972, subdivided into subjects without and with eosinophilia or positive skin tests

	Total		Eosinophilia				Positive skin tests			
			Subjects without		Subjects with		Subjects without		Subjects with	
	No.	%	No.	%	No.	%	No.	%	No.	%
All subjects										
Alive	4,172	78	3,698	78	474	72	3,460	76	712	85
Lost to follow-up	75	1	62	1	13	2	55	1	20	2
Deaths	1,135	21	960	20	175	26	1,033	23	102	12
Total subjects	5,382	100	4,720	100	662	100	4,548	100	834	100
All deceased subjects										
Cardiovascular death	507	45	418	44	89	51	463	45	44	43
Ischemic heart death	303	27	252	26	51	29	278	27	25	25
Nonischemic heart death	204	18	166	17	38	22	185	18	19	19
Cerebrovascular death	95	8	75	8	20	11	88	9	7	6
Other cardiovascular death	109	10	91	9	18	10	97	9	12	12
Other death causes*	628	55	542	56	86	49	570	55	58	57
Total deaths	1,135	100	960	100	175	100	1,033	100	102	100

* Including 10 deaths of unknown cause because subjects died abroad.

panel). The association between positive skin tests and cardiovascular mortality and its subgroups was limited to subjects with both positive skin tests and asthma who had an increased risk for death from other cardiovascular disease (RR = 7.65; 95 percent CI: 1.61, 36.4).

Further analyses revealed that the association between eosinophilia and cardiovascular mortality was not different for subgroups of sex, age, smoking habits, body mass index, and city. Table 4 shows that the association between eosinophilia and cardiovascular mortality was limited to subgroups with FEV_1 percent predicted < 100 percent: subjects with eosinophilia had a 1.10 (95 percent CI: 0.70, 1.73) increased risk in the subgroup with FEV_1 percent predicted ≥100 percent, a 2.07 (RR = 1.1 × 1.88; 95 percent CI: 1.48, 2.89) increased risk in the subgroup with FEV_1 percent predicted 80–99.99 percent, and a 2.12 increased risk (RR = 1.1 × 1.94; 95 percent CI: 1.32, 3.43) in the subgroup with FEV_1 percent predicted <80 percent compared with subjects without eosinophilia. Although not statistically significant, the same pattern was present for mortality from ischemic and cardiovascular diseases other than ischemic heart disease and, within the subgroup of cardiovascular diseases other than ischemic heart disease, in the group of other cardiovascular disease.

The association between positive skin tests and cardiovascular death was different within subgroups of FEV_1 percent predicted, body mass index, and smoking but not within subgroups of sex and age. Subjects with (near) normal lung function and body weight, that is, FEV_1 percent predicted ≥80 percent and body mass index 18.50–24.99 kg/m², who did not smoke had a significantly reduced risk for cardiovascular death (RR = 0.15; 95 percent CI: 0.05, 0.46), ischemic heart disease death (RR = 0.10; 95 percent CI: 0.02, 0.50), and death from other cardiovascular disease (RR = 0.11; 95 percent CI: 0.01, 0.99). Conversely, this risk increased by 8.3 (1.42 × 5.86), 3.8 (1.14 × 3.33), 10 (1.19 × 8.40), or 3.9 (1.55 × 2.50)-fold when subjects had low FEV_1 (<80 percent of predicted) or were overweight (body mass index 25.00–29.99), were obese (body mass index ≥30 kg/m²), or smoked, respectively. The same pattern was seen for mortality from ischemic and cardiovascular diseases other than ischemic heart disease and within the latter subgroup for death from other cardiovascular disease. Adjustment for the interaction between FEV_1 percent predicted and body mass index did not change these results.

Additional adjustment of the models in table 4 for asthma and its interactions with the allergy markers did not essentially change the associations of eosinophilia and the presence of positive skin tests with cardiovascular mortality. The relative risk for positive skin tests and asthma with mortality from other cardiovascular disease was reduced from 7.65 (95 percent CI: 1.61, 36.4; table 3) to 4.66 (95 percent CI: 0.83, 26.2) and was no longer significant after interactions of eosinophilia and positive skin tests with other risk factors were taken into account.

TABLE 3. Relative risks of cardiovascular mortality during 30 years of follow-up in relation to eosinophilia and positive skin tests in 1965–1972 in Cox regression in the Vlagtwedde-Vlaardingen Study (the Netherlands; $n = 5,382$), adjusted for major risk factors and without and with additional adjustment for a history of asthma attacks

	All cardiovascular deaths ($n = 507$)	All cardiovascular deaths		Nonischemic heart deaths	
		Ischemic ($n = 303$)	Nonischemic ($n = 204$)	Cerebrovascular ($n = 95$)	Other cardiovascular ($n = 109$)
*No adjustment for asthma **					
Eosinophilia	1.73 (1.37, 2.18)†,‡	1.64 (1.21, 2.23)‡	1.92 (1.34, 2.74)‡	2.30 (1.39, 3.79)‡	1.62 (0.97, 2.70)
Positive skin tests	1.03 (0.75, 1.42)	0.95 (0.62, 1.44)	1.16 (0.72, 1.88)	0.91 (0.42, 2.00)	1.40 (0.75, 2.59)
Male sex	1.60 (1.22, 2.09)‡	1.80 (1.26, 2.58)‡	1.35 (0.89, 2.04)	0.97 (0.53, 1.76)	1.79 (1.01, 3.17)‡
Age, per 10-year increase	3.46 (3.16, 3.84)‡	3.05 (2.71, 3.44)‡	4.44 (3.74, 5.27)‡	4.62 (3.60, 5.94)‡	4.30 (3.40, 5.45)‡
Smoking	1.61 (1.21, 2.14)‡	2.14 (1.46, 3.15)‡	1.11 (0.72, 1.70)	1.35 (0.72, 2.54)	0.94 (0.53, 1.69)
Exsmoking	1.36 (0.93, 2.01)	1.33 (0.77, 2.29)	1.48 (0.85, 2.57)	2.09 (0.95, 4.61)	1.10 (0.51, 2.38)
FEV_1, % predicted§					
≥100%	1	1	1	1	1
80–99.99%	1.20 (0.99, 1.46)	1.22 (0.96, 1.57)	1.15 (0.84, 1.57)	1.15 (0.73, 1.79)	1.15 (0.75, 1.77)
<80%	1.82 (1.42, 2.34)‡	1.56 (1.11, 2.18)‡	2.26 (1.55, 3.29)‡	1.76 (0.98, 3.13)	2.75 (1.67, 4.53)‡
Body mass index (kg/m^2)					
<18.50	2.34 (0.57, 9.52)	0.00 (0.00, ∞)	8.25 (1.96, 34.7)‡	9.09 (1.19, 69.7)‡	7.59 (1.00, 57.6)‡
18.50–24.99	1	1	1	1	1
25.00–29.99	1.25 (1.02, 1.54)‡	1.41 (1.08, 1.84)‡	1.03 (0.74, 1.42)	0.94 (0.59, 1.49)	1.12 (0.72, 1.75)
≥30	1.40 (1.05, 1.88)‡	1.72 (1.19, 2.51)‡	1.03 (0.65, 1.63)	0.80 (0.41, 1.56)	1.29 (0.70, 2.40)
City	1.02 (0.86, 1.22)	0.82 (0.65, 1.04)	1.43 (1.07, 1.89)‡	0.96 (0.63, 1.44)	2.04 (1.36, 3.06)‡
With additional adjustments for asthma and its interactions with the allergy markers ¶					
Eosinophilia	1.63 (1.28, 2.09)‡	1.63 (1.19, 2.25)‡	1.69 (1.14, 2.51)‡	2.10 (1.23, 3.57)‡	1.36 (0.75, 2.44)
Positive skin tests	0.95 (0.68, 1.33)	0.93 (0.60, 1.43)	0.97 (0.57, 1.67)	0.97 (0.44, 2.13)	0.98 (0.47, 2.06)
Asthma	0.75 (0.44, 1.29)	0.86 (0.44, 1.67)	0.63 (0.25, 1.59)	0.31 (0.04, 2.26)	0.85 (0.28, 2.58)
Eosinophilia and asthma	1.73 (0.78, 3.87)	1.11 (0.36, 3.48)	2.55 (0.74, 8.75)	6.13 (0.59, 64.3)	1.87 (0.42, 8.40)
Positive skin tests and asthma	2.38 (0.89, 6.34)	1.40 (0.29, 6.70)	3.79 (1.00, 14.4)		7.65 (1.61, 36.4)‡

* Values are the result when adjusted for eosinophilia, positive skin tests, sex, age, smoking habits, FEV_1 % predicted, body mass index, and city.

† Numbers in parentheses, 95% confidence interval.

‡ Significant at $p < 0.05$.

§ FEV_1 % predicted, percentage of predicted forced expiratory volume in 1 second.

¶ Values are the result when adjusted for eosinophilia, positive skin tests, sex, age, smoking habits, FEV_1 % predicted, body mass index, city, and asthma and the interactions of the allergy markers with asthma.

DISCUSSION

To our knowledge, this is the first large-scale study showing that eosinophilia is significantly associated with increased risk for cardiovascular death (RR = 1.7; 95 percent CI: 1.4, 2.2), not only with death from ischemic heart disease (RR = 1.6; 95 percent CI: 1.2, 2.2) but also with cerebrovascular disease death (RR = 2.3; 95 percent CI: 1.4, 3.8). This increased risk was restricted to subjects with a low or (near) normal lung function, that is, an FEV_1 <100 percent of predicted. Subjects with positive skin tests had a decreased risk of mortality from cardiovascular disease when they had normal weight and FEV_1 percent predicted ≥80 percent and did not smoke. In contrast, subjects with positive skin tests had an increased risk for cardiovascular mortality when they had one or more risk factors for cardiovascular mortality, that is, increased body mass index, FEV_1 percent predicted <80 percent, or smoking. Furthermore, these associations were present independent of a history of asthma attacks.

As far as we know, there is no literature on the relation between eosinophilia and cardiovascular *death*. Three studies have observed a higher *incidence* of ischemic heart disease with eosinophilia, including the first event of angina pectoris, myocardial infarction, or death due to ischemic heart disease (9, 10, 12). These studies were all restricted to the *incidence* of ischemic heart disease within 5 years of follow-up. Our study extends these observations in that the association between eosinophilia and *death* due to ischemic heart disease is present during a maximum of 30 years of follow-up and after adjustment for other major risk factors for cardiovascular mortality. Thus, eosinophils may play a role in not only the incidence of but also the mortality from cardiovascular disease.

In this study eosinophilia was not only related to ischemic heart death but also strongly to cerebrovascu-

TABLE 4. Relative risks of cardiovascular mortality during 30 years of follow-up in relation to eosinophilia and positive skin tests in Cox regression in the Vlagtwedde-Vlaardingen Study (the Netherlands; n = 5,382), adjusted for major risk factors and additional adjustment for the interactions of the allergy markers with these risk factors

Variable	No.	All cardiovascular deaths (n = 507)	All cardiovascular deaths		Nonischemic heart deaths	
			Ischemic (n = 303)	Nonischemic (n = 204)	Cerebrovascular (n = 95)	Other cardiovascular (n = 109)
Eosinophilia (EO+)	562	1.10 (0.70, 1.73)*,†	1.09 (0.61, 1.95)	1.15 (0.55, 2.41)	1.65 (0.64, 4.26)	0.77 (0.24, 2.52)
Positive skin tests (ST+)	634	0.15 (0.05, 0.46)‡	0.10 (0.02, 0.50)‡	0.20 (0.04, 1.05)	0.43 (0.03, 6.33)	0.11 (0.01, 0.99)‡
Male sex	2,920	1.62 (1.24, 2.13)‡	1.85 (1.29, 2.66)‡	1.35 (0.89, 2.04)	0.96 (0.52, 1.75)	1.82 (1.02, 3.23)‡
Age, per 10-year increase		3.56 (3.22, 3.93)‡	3.11 (2.76, 3.50)‡	4.54 (3.81, 5.41)‡	4.63 (3.59, 5.96)‡	4.52 (3.54, 5.76)‡
Smoking	2,982	1.55 (1.16, 2.07)‡	2.10 (1.42, 3.12)‡	1.04 (0.67, 1.61)	1.28 (0.67, 2.44)	0.88 (0.48, 1.59)
Exsmoking	475	1.28 (0.86, 1.92)	1.31 (0.75, 2.30)	1.34 (0.75, 2.39)	2.05 (0.91, 4.64)	0.91 (0.39, 2.09)
FEV_1, % predicted§						
≥100%	2,628	1	1	1	1	1
80–99.99%	2,361	1.11 (0.89, 1.38)	1.14 (0.87, 1.50)	1.05 (0.73, 1.48)	1.08 (0.64, 1.81)	1.02 (0.63, 1.67)
<80%	493	1.42 (1.06, 1.91)‡	1.11 (0.74, 1.66)	1.98 (1.29, 3.05)‡	1.81 (0.94, 3.48)	2.11 (1.19, 3.76)‡
BMI§ (kg/m²)						
<18.50	94	2.52 (0.62, 10.3)	0.00 (0.00, ∞)	9.12 (2.17, 38.3)‡	10.1 (1.32, 77.9)‡	6.35 (1.10, 63.3)‡
18.50–24.99	2,719	1	1	1	1	1
25.00–29.99	2,044	1.14 (0.92, 1.41)	1.27 (0.96, 1.66)	0.95 (0.68, 1.33)	0.85 (0.58, 1.55)	0.95 (0.60, 1.53)
≥30	525	1.19 (0.88, 1.61)	1.45 (0.98, 2.14)	0.89 (0.56, 1.43)	0.75 (0.38, 1.51)	1.04 (0.54, 1.97)
City	2,406	1.04 (0.87, 1.25)	0.84, 0.67, 1.06)	1.45 (1.09, 1.93)‡	0.96 (0.63, 1.45)	2.09 (1.39, 3.13)‡
EO+ and FEV_1 % predicted ≥100%	259	1	1	1	1	1
EO+ and FEV_1 % predicted 80–99.99%	306	1.88 (1.07, 3.31)‡	1.59 (0.77, 3.31)	2.32 (0.95, 5.67)	1.96 (0.60, 6.38)	2.62 (0.70, 11.4)
EO+ and FEV_1 % predicted <80%	97	1.94 (1.00, 3.75)‡	2.20 (0.93, 5.21)	1.63 (0.58, 4.59)	1.08 (0.24, 4.80)	2.61 (0.58, 11.8)
ST+ and smoking	490	2.50 (1.01, 6.16)‡	1.80 (0.55, 5.86)	4.09 (0.93, 17.1)	4.07 (0.34, 49.3)	3.85 (0.67, 22.1)
ST+ and exsmoking	79	2.85 (0.91, 8.97)	1.46 (0.26, 8.39)	5.57 (1.09, 28.6)‡	3.43 (0.16, 74.3)	6.89 (0.96, 49.4)
ST+ and FEV_1 % predicted ≥100%	403	1	1	1	1	1
ST+ and FEV_1 % predicted 80–99.99%	361	1.01 (0.48, 2.13)	1.40 (0.50, 3.90)	0.66 (0.21, 2.01)	0.38 (0.06, 2.25)	1.02 (0.23, 4.48)
ST+ and FEV_1 % predicted <80%	70	5.86 (2.62, 13.1)‡	10.6 (3.66, 31.8)‡	2.96 (0.86, 10.1)	1.01 (0.10, 10.3)	5.38 (1.13, 25.6)‡
ST+ and BMI <18.50 kg/m²	18	0.00 (0.00, ∞)	5.17 (0.00., ∞)	0.00 (0.00, ∞)	0.00 (0.00, ∞)	0.00 (0.00, ∞)
ST+ and BMI 18.50–24.99 kg/m²	485	1	1	1	1	1
ST+ and BMI 25.00–29.99 kg/m²	271	3.33 (1.58, 7.02)‡	4.98 (1.68, 14.6)‡	2.38 (0.82, 6.96)	0.78 (0.13, 4.69)	5.00 (1.15, 21.7)‡
ST+ and BMI ≥30 kg/m²	50	8.40 (2.97, 23.8)‡	10.8 (2.52, 44.5)‡	7.64 (1.57, 37.0)‡	4.90 (0.36, 66.3)	10.2 (1.28, 81.3)‡

* Values are the result when adjusted for eosinophilia, positive skin tests, sex, age, smoking habits, FEV_1 % predicted, body mass index, city, and the interactions presented.
† Numbers in parentheses, 95% confidence interval.
‡ Significant at $p < 0.05$.
§ FEV_1 % predicted, percentage of predicted forced expiratory volume in 1 second; BMI, body mass index.

lar death. The relation between eosinophilia and death from cerebrovascular disease has never been studied before. It is important to realize that, when we studied cerebrovascular death, we used all deaths due to cerebrovascular disease as the endpoint of interest (95 of 507 cardiovascular deaths) and considered all 412 other cardiovascular deaths as censored. Thus, the reference group included all subjects still alive, lost to follow-up, and dead from causes other than cerebrovascular disease, for example, cancer and ischemic heart disease. Therefore, the increased relative death risk of 2.2 due to cerebrovascular disease is even underestimated, because there were subjects with eosinophilia who had already died from ischemic heart disease.

The predictive relation of a single risk factor, for example, eosinophilia, for cardiovascular mortality may reflect its association with other risk factors. It has long been recognized that the established risk factors male sex, older age, smoking (11), hypertension, hypercholesterolemia (1–4), overweight (3), and total leukocyte count (31–33) are associated with increased cardiovascular mortality, as well as low lung function (5, 6). Data on hypercholesterolemia and hypertension were not available in this cohort study, yet that seems of little importance since we found no evidence in the literature that eosinophilia is associated with hypercholesterolemia or hypertension. In contrast, male sex, smoking, and low lung function (13, 14, 22) are all associated with eosinophilia. In this study, eosinophilia remained a risk factor, after controlling for smoking, only in subjects with an FEV_1 <100 percent-of-predicted.

Since the total leukocyte count is not available, we cannot rule out that the associations are a reflection of the known association of total leukocyte counts with cardiovascular disease (31–33). In the 1960s the interest of the researchers focused on eosinophilia as an allergy marker and not as a part of the total leukocyte count, and they measured only the absolute eosinophil count. Only in the last follow-up in 1989/1990 of the young cohort, aged 15–39 years at the initial survey in 1967/1969, was the total leukocyte count also measured. The correlation coefficient between eosinophils and total leukocyte count was 0.24, which means that 0.78 of the variation in eosinophils was not explained by its association with total leukocyte counts. In addition, Prentice et al. (9) showed that, based on 79,274 total leukocyte and differential determinations, the average eosinophil fraction of the total leukocyte count was 2.8 percent. Since the eosinophil count constitutes only such a small fraction of the total leukocyte count, elevated eosinophil counts need not influence the total leukocyte count, and an increased total leukocyte count need not be caused by an increased eosinophil count. Therefore, we think that our results are not just a reflection of the association of total leukocyte count with cardiovascular disease.

Because of the relation between eosinophilia and both ischemic heart disease mortality and mortality from cerebrovascular disease, it seems plausible that eosinophils have a role in the endothelial inflammation of atherosclerosis. We suggest two potential mechanisms. First, Marone (34) proposed the mechanism that eosinophils can secrete among others eosinophil cationic protein and major basic protein, which can activate cardiac mast cells. These activated mast cells can then release histamine, which can result in coronary artery spasm and arrhythmias. This theory is in agreement with those of Hällgren et al. (35, 36), who suggested the active participation of eosinophils in the inflammatory process in patients with acute myocardial infarction, and Trillo (37), who found eosinophils in the aortic fatty streaks of African green monkeys. Second, the enzyme arachidonate 15-lipoxygenase is expressed in significant quantities in eosinophils (38, 39). This enzyme may be involved in oxidative modification of low density lipoprotein in the early phase of atherogenesis and may contribute to the development of atherosclerotic lesions (40).

Subjects with positive skin tests, normal body weight, and (near) normal lung function who did not smoke had a decreased risk for total cardiovascular mortality. However, when subjects with positive skin tests had an accumulation of other risk factors for cardiovascular mortality, especially obesity, low levels of lung function, or smoking, they had an increased risk of cardiovascular mortality. This is the first time that the relation between positive skin tests and mortality from cardiovascular disease has been investigated, and the results need confirmation. Rozencwaig (7) reported an increased *incidence* of heart disease in 50 allergic patients with positive allergy tests compared with 50 nonallergic patients. However, he did not mention explicitly that respiratory allergy was studied, and the results were not adjusted for lung function, body mass index, and smoking habits. His patients most likely had not only positive allergy tests but other risk factors for cardiovascular mortality that may have driven the results. We found that the presence of positive skin tests was not associated with cardiovascular mortality, after adjustment for its major risk factors (table 3). This result is probably due to the high prevalence of overweight and obesity (almost 50 percent) and smoking (55 percent) in this population (table 1). We can only speculate as to the mechanisms behind these associations. One explanation might be that positive skin tests are associated with other well-known risk factors for cardiovascular mortality for which we did not adjust in this study, that is, hypertension, hypercholesterolemia, physical inactivity, psychosocial factors, and diabetes mellitus. To our knowledge, however, there is no evidence of increased prevalence of these risk factors in subjects with positive skin tests.

Our study showed that body mass index, a well-known risk factor for cardiovascular mortality (3), was not predictive by itself after taking into account its interaction with the presence of positive skin tests (table 4). Furthermore, it seems that the association between impaired lung function and cardiovascular disease, which has been reported several times (5, 6), was partly mediated by the presence of allergy, manifested as both positive skin tests and eosinophilia. These observations point out new areas for research in the pathogenesis of cardiovascular disease for manifestations of allergy in combination with well-known risk factors for cardiovascular mortality.

In conclusion, our data suggest a possible link between the allergy markers, peripheral blood eosinophilia and positive skin tests, and cardiovascular mortality, both mortality of ischemic heart disease and cerebrovascular disease. These increased risks were present in combination with other risk factors, that is, reduced lung function in combination with eosinophilia and positive skin tests, and overweight and smoking in combination with positive skin tests. Weight reduction (41) and smoking cessation (42) have been shown to decrease the risk for cardiovascular death, and our results suggest that this can be especially important in individuals with positive skin tests. Nonallergic causes of eosinophilia seem to play an important role since the...

association remained unchanged after adjustment for positive skin tests and asthma.

Since allergy increases in Western countries, our findings may have important implications for health economics and future mortality statistics. We recommend further research into the role of these allergy markers in the pathogenesis of cardiovascular disease to elucidate mechanisms for these epidemiologic associations.

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APPENDIX

ICD Codes Used during the Years 1965–1995

Ischemic heart disease: ICD-7 codes 420 and 422.1; ICD-8 codes 410–414; ICD-9 codes 410–414 and 429.2; *cerebrovascular disease*: ICD-7 codes 330–334; ICD-8 codes 430–438; ICD-9 codes 430–438; *other heart disease*: ICD-7 codes 422 (but not 422.1), 431–434, and 782.4; ICD-8 codes 420, 422–426 (but not 424), 427–429, and 782.4; ICD-9 codes 416, 420, 422, 423, 425–428, and 429 (but not 429.2); *diseases of arteries*: ICD-7 codes 450–456; ICD-8 codes 440–448 (but not 444.2); ICD-9 codes 440–448 and 785.4; *rheumatic heart disease*: ICD-7 codes 400–416 and 421; ICD-8 codes 390–398 and 424; ICD-9 codes 390–398 and 424; *hypertensive disease*: ICD-7 codes 440–447; ICD-8 codes 400–404; ICD-9 codes 401–405; *other diseases of vascular system*: ICD-7 codes 460–468 (but not 465); ICD-8 codes 451–458; ICD-9 codes 417 and 451–459; *pulmonary embolism*: ICD-7 code 465; ICD-8 code 450; ICD-9 code 415; and *(sub)acute endocarditis*: ICD-7 code 430; ICD-8 code 421; ICD-9 code 421.

APPENDIX B

Invited Review

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Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology

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Li, Jian-Mei, and Ajay M. Shah. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* 287: R1014–R1030, 2004; doi:10.1152/ajpregu.00124.2004.—The endothelial generation of reactive oxygen species (ROS) is important both physiologically and in the pathogenesis of many cardiovascular disorders. ROS generated by endothelial cells include superoxide ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{-}\cdot$), nitric oxide (NO), and hydroxyl ($\cdot OH$) radicals. The $O_2^-\cdot$ radical, the focus of the current review, may have several effects either directly or through the generation of other radicals, e.g., H_2O_2 and $ONOO^{-}\cdot$. These effects include *1*) rapid inactivation of the potent signaling molecule and endothelium-derived relaxing factor NO, leading to endothelial dysfunction; *2*) the mediation of signal transduction leading to altered gene transcription and protein and enzyme activities ("redox signaling"); and *3*) oxidative damage. Multiple enzymes can generate $O_2^-\cdot$, notably xanthine oxidase, uncoupled NO synthase, and mitochondria. Recent studies indicate that a major source of endothelial $O_2^-\cdot$ involved in redox signaling is a multicomponent phagocyte-type NADPH oxidase that is subject to specific regulation by stimuli such as oscillatory shear stress, hypoxia, angiotensin II, growth factors, cytokines, and hyperlipidemia. Depending on the level of oxidants generated and the relative balance between pro- and antioxidant pathways, ROS may be involved in cell growth, hypertrophy, apoptosis, endothelial activation, and adhesivity, for example, in diabetes, hypertension, atherosclerosis, heart failure, and ischemia-reperfusion. This article reviews our current knowledge regarding the sources of endothelial ROS generation, their regulation, their involvement in redox signaling, and the relevance of enhanced ROS generation and redox signaling to the pathophysiology of cardiovascular disorders where endothelial activation and dysfunction are implicated.

antioxidant: NADPH oxidase; oxidative stress; reactive oxygen species; redox signaling

THE ENDOTHELIAL LINING of blood vessels and the heart is an active tissue that plays a pivotal role in maintaining cardiovascular homeostasis, including important functions such as the regulation of vascular tone and tissue perfusion, vascular permeability, myocardial function, blood fluidity, anticoagulant activity, and inflammatory responses (18, 137). Endothelial cells, located at the interface between blood and tissue, can sense changes in hemodynamic forces, ambient P_{O_2}, and local blood-borne signals and respond with appropriate changes in function to maintain homeostasis. These responses include *1*) the paracrine release of diffusible mediators such as nitric oxide (NO), prostacyclin, endothelin-1 (ET-1), and growth factors; *2*) the activity of surface enzymes such as ACE, which regulates local levels of bioactive angiotensin II and bradykinin; and *3*) the expression of surface proteins such as adhesion molecules that interact with other cell types. "Activation" of endothelial cells is associated with a phenotype that promotes the recruitment of inflammatory cells to sites of vascular injury, but may also result in increased vascular permeability and intravascular thrombosis. Chronic endothelial dysfunction is implicated in the pathogenesis of several diseases including atherosclerosis, hypertension, diabetic vasculopathy, and heart failure (18).

Endothelial cells generate reactive oxygen species (ROS), including superoxide ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), NO, peroxynitrite ($ONOO^{-}\cdot$), hydroxyl radicals ($\cdot OH$), and other radicals (29). The physiological functions of endothelial NO are well recognized, have been comprehensively reviewed (18, 138), and will not be considered in detail herein, except inasmuch as they are affected by reaction with other ROS (notably $O_2^-\cdot$). More recently, it has become clear that ROS such as $O_2^-\cdot$ and H_2O_2 also have several potentially important effects on endothelial function and phenotype and are implicated both in physiological regulation and disease pathophysiology. $O_2^-\cdot$ production usually involves a one-electron reduction of molecular O_2. The negatively charged $O_2^-\cdot$ radical is unstable in aqueous solution (half-life of a few seconds) and is rapidly dismutated to H_2O_2. It is poorly cell membrane permeable and is generally restricted to the cell compartment in which it is produced. It can undergo several chemical reactions depending on the amount generated and the localization and proximity to other radicals and enzymes. $O_2^-\cdot$ reacts rather

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poorly with itself to produce H_2O_2 and O_2 (rate constant 8×10^4 mol·l^{-1}·s^{-1}), but this reaction is substantially accelerated (rate constant $\sim 2 \times 10^9$ mol·l^{-1}·s^{-1}) by superoxide dismutase (SOD). Thus, when $O_2^{-}\cdot$ production is relatively low (picomolar range), most biological effects are likely to be secondary to H_2O_2 production. Indeed, H_2O_2 is more stable and diffusible than $O_2^{-}\cdot$ and is more cell membrane permeable and may therefore be more relevant than $O_2^{-}\cdot$ with respect to modulation of signal transduction pathways. $O_2^{-}\cdot$ reacts with NO at a significantly faster rate than with SOD (rate constant $\sim 7 \times 10^9$ mol·l^{-1}·s^{-1}), so that when levels of NO are in the high nanomolar range, NO may outcompete SOD and react with $O_2^{-}\cdot$ to generate $ONOO\cdot^{-}$, this reaction also resulting in NO inactivation. When $O_2^{-}\cdot$ levels are higher still, it can react with iron-sulfur centers in many proteins and can also release iron, which reacts with H_2O_2 to produce the highly reactive ·OH radical; these last reactions are often involved in oxidative stress-associated tissue injury. In summary, $O_2^{-}\cdot$ may *1*) serve as a precursor for other ROS such as H_2O_2 and thereby act as a regulatory mediator in signaling processes leading to altered gene transcription and protein and enzyme activities (so-called "redox signaling"); *2*) rapidly inactivate NO, thereby causing endothelial dysfunction; *3*) cause oxidative damage of macromolecules, membranes, and DNA usually indirectly through the generation of more toxic (reactive) radicals such as $ONOO\cdot^{-}$ and ·OH. Redox signaling secondary to tightly regulated ROS production by specific enzymes and the ROS-dependent inactivation of NO are fundamentally important mechanisms in the pathogenesis of several cardiovascular disorders. In this article, we review current knowledge regarding the sources of ROS generation in endothelial cells, their regulation and involvement in redox signaling, and the relevance of enhanced endothelial ROS generation and redox signaling for cardiovascular pathophysiology.

ENDOTHELIAL SOURCES OF $O_2^{-}\cdot$

Potential sources of endothelial $O_2^{-}\cdot$ generation that are implicated in disease processes include mitochondria, xanthine oxidase (XO), uncoupled NO synthases, cytochrome *P*-450 enzymes, and NADPH oxidases. In addition, enzymes such as lipoxygenases may also generate $O_2^{-}\cdot$ (29).

Mitochondria. The mitochondrial respiratory chain can be a major source of $O_2^{-}\cdot$, which may then be converted to H_2O_2. During aerobic metabolism, the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP via a multicomponent NADH dehydrogenase complex (92). Molecular O_2 serves as the final electron acceptor for cytochrome (Cyt)-*c* oxidase (complex IV), the terminal component of the respiratory chain, and is ultimately reduced to H_2O. Up to 1–4% of O_2 may be incompletely reduced, resulting in $O_2^{-}\cdot$ formation, mainly at complex I (NADH coenzyme Q reductase) and complex III (ubiquinol Cyt *c* reductase). In the presence of transition metal ions, ·OH radicals may also be formed (92).

Increased mitochondrial $O_2^{-}\cdot$ generation in endothelial cells appears to be particularly prominent in situations of metabolic perturbation. For example, hyperglycemia induces mitochondrial $O_2^{-}\cdot$ production, which has been shown to contribute to the activation of the hexosamine pathway and be involved in the pathogenesis of diabetic complications (31). Similarly, the adipokine leptin (which is involved in the regulation of body adiposity and weight) induces mitochondrial $O_2^{-}\cdot$ production in cultured bovine aortic endothelial cells by increasing fatty acid oxidation via protein kinase A (171). Other settings in which mitochondrial-derived $O_2^{-}\cdot$ radicals are increased include hypoxia-reoxygenation and ischemia-reperfusion, where the enhanced $O_2^{-}\cdot$ is at least partially responsible for an increase in endothelial permeability (127).

XO. Xanthine oxidoreductase (XOR) is a ubiquitous metalloflavoprotein found as one of two interconvertible yet functionally distinct forms, namely xanthine dehydrogenase (XD), which is constitutively expressed in vivo, and XO, which is generated by the posttranslational modification of XD (114). Functionally, both XD and XO catalyze oxidation of hypoxanthine to xanthine and xanthine to urate (114). However, whereas XD requires NAD^+ as an electron acceptor, XO instead requires the reduction of molecular O_2, thereby generating $O_2^{-}\cdot$. The conversion of XD to XO occurs either through reversible thiol oxidation of sulfhydryl residues on XD or via irreversible proteolytic cleavage of a segment of XD during hypoxia, ischemia, or in the presence of various proinflammatory mediators [e.g., tumor necrosis factor-α (TNF-α)] (114, 145). Of note, the former pathway provides a mechanism whereby XO activity may increase further in response to oxidative stress. Interestingly, XO may exist in a molybdenum-deficient form, in which state it is unable to use xanthine as a substrate but can nevertheless generate $O_2^{-}\cdot$ at the expense of NADH. This state is relevant experimentally, as in this case $O_2^{-}\cdot$ generation is not inhibited by XO inhibitors such as oxypurinol but is inhibited by the flavoprotein inhibitor diphenyleneiodonium (DPI) (175).

XOR is expressed at high levels on the luminal surface of the endothelium of many organs including the human heart. Its expression may be transcriptionally upregulated by cytokines such as interferon-γ, although XO activity appears to be regulated mainly through the posttranslational pathways described earlier (18, 114). An area of controversy has been the apparent paradox that XO-mediated $O_2^{-}\cdot$ production (usually assessed by inhibition by allopurinol or oxypurinol) can be documented in several pathophysiological conditions in organs where there is apparently very low or undetectable constitutive XOR activity. There are several possible explanations for this finding: *1*) endothelial XO expression may be diluted and underestimated in assays of whole organs, *2*) oxypurinol and allopurinol can directly scavenge ROS in some settings (i.e., their effects would not be specific for XO), and *3*) it appears that XO produced in XOR-rich organs can be mobilized into the systemic circulation and then bind to endothelial cells at distant sites in a heparin-reversible manner (114).

A large body of evidence supports an important role for XO-mediated ROS generation in tissue injury during reperfusion, although there may be a relatively narrow window in which this can be therapeutically targeted (102). Cell culture studies using the ROS-generating XO system indicate that H_2O_2 may be the key promoter of tissue injury in this setting (83). Secondary to XO activation, the local accumulation and activation of neutrophils may also significantly enhance local ROS production (e.g., via neutrophil NADPH oxidase and via myeloperoxidase-mediated ROS generation). From a physiological perspective, endothelial XO-mediated ROS generation may serve as a mechanism that recruits and activates neutro-

phils as part of the microvascular inflammatory response to pathogens (114). Indeed, XO is activated by proinflammatory mediators such as TNF-α, interleukins, complement 5a, and lipopolysaccharide. Increased $O_2^{\cdot-}$ production from XO has also been found both experimentally and clinically in several disease settings and may contribute to the genesis of vascular endothelial dysfunction as well as redox signaling, leading to altered gene/protein expression (see INVOLVEMENT OF INCREASED ENDOTHELIAL $O_2^{\cdot-}$ GENERATION IN CARDIOVASCULAR DISEASE).

Cytochrome P-450. Cytochrome *P*-450 (CYP) enzymes have traditionally been recognized as heme-containing hepatic endoplasmic reticular flavoenzymes that can oxidize, peroxidize, and/or reduce cholesterol, vitamins, steroids, and many other compounds in an oxygen- and NADPH-dependent manner (40). More recently, it has become clear that specific CYP enzymes are also expressed in extrahepatic tissues, including the cardiovasculature. CYP enzymes that metabolize arachidonic acid (namely the CYP2 and CYP4A gene families) are implicated in vascular regulation through the generation of vasodilator metabolites, vasoconstrictors, and ROS (40). For example, the compound 20-HETE generated by CYP4A in vascular smooth muscle cells is implicated in the myogenic response. Fleming and colleagues (39, 40) showed that CYP2 epoxygenases expressed on endothelial cells (notably CYP2C8/9) generate epoxyeicosatrienoic acids (EETs) that account for the NO- and prostacyclin-independent endothelium-dependent hyperpolarizing factor (EDHF) activity responsible for vasodilatation in several vascular beds, including the heart and kidney (39, 40). Like all CYP enzymes, CYP2C enzymes are inhibited by NO, leading to suggestions that CYP2C-mediated vasodilator activity is only of significance in settings where NO bioavailability is reduced.

Recently, it has been appreciated that vascular CYP enzymes can also generate $O_2^{\cdot-}$, H_2O_2, and ·OH during the CYP reaction cycle when the electrons for the reduction of the central heme iron are transferred to the activated bound O_2 molecule in an NADPH-dependent reaction (40). Thus the CYP2C involved in the EDHF response in porcine coronary arteries was identified as a significant source of ROS in cultured and native endothelial cells (41). Because CYP2C enzymes can generate both EDHF (a vasodilator) and ROS (which are potentially vasoconstrictor through inactivation of NO or vasodilator through conversion to H_2O_2), the effects of altered CYP2 activity may be quite complex. In endothelial cells, CYP2C-derived $O_2^{\cdot-}$ impaired NO-dependent vascular relaxation and elevated redox-sensitive nuclear factor (NF)-κB activity and VCAM-1 expression (41). Likewise, TNF-α-induced endothelial cell adhesion molecule expression was attenuated by several CYP inhibitors, e.g., ketoconazole (135). In humans with coronary artery disease, however, CYP2C9 inhibition with sulphafenazole improved endothelium-dependent, NO-mediated vasodilatation, probably by reducing ROS production (37).

Endothelial CYP activity and expression are stimulated by cyclic stretch, hormonal stimuli, and HMG-CoA reductase inhibitors (statins) (39), leading to both increased ROS and increased EDHF. Pathological conditions in which CYP expression has been reported to increase include hypertension and hypercholesterolemia. In the case of statins, which also increase endothelial NOS (eNOS) expression, it is feasible that there is increased potential to generate $OONO^{\cdot-}$ (from reaction of NO and $O_2^{\cdot-}$). On the other hand, oxidized LDL reduces endothelial CYP2 family expression via ROS probably generated by NADPH oxidase(s) and acting through reduced expression of the transcriptional regulator NF-1 (151).

Dysfunctional or uncoupled NOSs. eNOS is a calcium-dependent flavoenzyme that generates NO in a process that involves the oxidation of the amino acid L-arginine by the reduction of molecular O_2 (18). NOSs are complex homodimeric oxidoreductases that shuttle electrons from the reductase domain of one monomer (a CYP-like region containing the cofactors FAD, FMN, and NADPH) to the oxidase domain in the other subunit that contains the heme active site. In view of this enzymatic structure, it is not surprising that NOS can become "uncoupled," leading to the generation of $O_2^{\cdot-}$ rather than NO. The essential NOS cofactor tetrahydrobiopterin (BH_4) appears to have a key role in regulating NOS function by "coupling" the reduction of molecular O_2 to L-arginine oxidation as well as maintaining the stability of NOS dimers (152, 158). Thus BH_4 availability may be a crucial factor in the balance between NO and $O_2^{\cdot-}$ generation by eNOS. Furthermore, BH_4 itself is highly susceptible to oxidative degradation, and the initial oxidative loss of BH_4 in response to increased ROS production by NADPH oxidases has been shown to amplify oxidative stress through the resulting loss of NO production and increased NOS-dependent $O_2^{\cdot-}$ generation (86). Likewise, peroxynitrite (a product of the reaction between NO and $O_2^{\cdot-}$) may also oxidize BH_4 and represent another pathogenic cause of eNOS uncoupling (91). In addition to increased catabolism or degradation, another reason for BH_4 depletion may be its reduced synthesis. Biosynthesis of BH_4 occurs either via a de novo pathway in which GTP cyclohydrolase I is a rate-limiting step or via a so-called salvage pathway that uses sepiapterin as an intermediate step. The precise levels of BH_4 in vivo at which eNOS becomes uncoupled and therefore supports $O_2^{\cdot-}$ production remain unclear, but it is suggested that the ratio between reduced and oxidized BH_4 metabolites may be a key regulator of ROS production by eNOS (4b, 158). The possible mechanisms involved have been reviewed elsewhere (158). A deficiency of the NOS substrate, L-arginine, can also result in $O_2^{\cdot-}$ generation by the enzyme.

Elevated $O_2^{\cdot-}$ production from uncoupled NOS has been implicated in the pathophysiology of several disorders such as atherosclerosis, diabetes, hypertension, and hypercholesterolemia (see INVOLVEMENT OF INCREASED ENDOTHELIAL $O_2^{\cdot-}$ GENERATION IN CARDIOVASCULAR DISEASE).

NADPH oxidases. In recent years, it has become apparent that endothelial cells and other nonphagocytic cells constitutively express an $O_2^{\cdot-}$-generating enzyme analogous to the phagocyte NADPH oxidase of neutrophils (7, 12–14, 47, 50, 74, 88, 99). The prototypic neutrophil NADPH oxidase comprises a membrane-associated low-potential cytochrome b_{558} composed of one $p22^{phox}$ and one $gp91^{phox}$ subunit and several cytosolic regulatory subunits ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and the small G protein Rac1 or Rac2) that translocate to the membrane and associate with the cytochrome b_{558} on neutrophil activation (8). The latter process rapidly activates the oxidase, which is normally dormant in resting neutrophils, to generate large amounts of $O_2^{\cdot-}$ [of the order of 10 $nmol \cdot min^{-1} \cdot 10^6$ $cells^{-1}$ (88)] in a process that requires

NADPH as cofactor and is essential for nonspecific host defense.

All the classical neutrophil oxidase components are expressed in endothelial cells (12, 47, 74, 96, 97, 99), but the enzyme nevertheless exhibits several major differences from the neutrophil oxidase: *1*) it continuously generates a low level of O_2^-· even in unstimulated cells, although its activity can be further increased by several agonists; *2*) a substantial proportion of the O_2^-· generated by the enzyme is produced intracellularly, whereas neutrophil oxidase O_2^-· generation occurs mainly in the extracellular compartment. Recently we reported that a substantial proportion of NADPH oxidase subunit expression and functional activity in cultured endothelial cells is intracellular rather than plasma membrane bound. Furthermore, a significant proportion of the NADPH oxidase subunits in unstimulated cells is present as fully preassembled and functional ROS-generating complexes associated with the intracellular cytoskeleton, particularly in a perinuclear distribution (12, 97). These findings, which have been confirmed by other groups (42, 153), provide a potential explanation for the aforementioned differences between neutrophil and endothelial cell NADPH oxidases.

In the last 3–4 years, several isoforms of $gp91^{phox}$, each encoded for by separate genes and termed Noxs, have been identified in nonphagocytic cells (84, 88). To date, the Nox family comprises five members (Nox1–5), of which Nox2 is $gp91^{phox}$ or the neutrophil isoform. As mentioned above, Nox2 ($gp91^{phox}$) is expressed in endothelial cells, and evidence of a functional role for this isoform in phorbol ester-induced O_2^-· generation and endothelium-dependent relaxation has been demonstrated in studies with $gp91^{phox-/-}$ mice (47). Nox2 is also expressed in cardiomyocytes, fibroblasts, and vascular smooth muscle of human resistance arteries (14, 126, 153). Significant levels of Nox4 mRNA (indeed, apparently greater than the expression of Nox2 mRNA) are also detectable in endothelial cells, and a recent study that used Nox4 antisense oligonucleotides suggested that an Nox4-dependent oxidase contributes functionally to basal O_2^-· generation in endothelial cells (3). Nevertheless, the relative roles of these two oxidases in endothelial cells remain to be fully elucidated. Recent studies have also reported the expression of homologues of $p47^{phox}$ and $p67^{phox}$, termed NOXO1 (Nox organizing protein 1; $p41^{nox}$) and NOXA1 (Nox activating protein 1, $p51^{nox}$) respectively, in colonic epithelium (9, 150), but their expression in endothelial cells has not been documented. The structure, function, and biological relevance of vascular NADPH oxidases and the potential roles of these homologues have been comprehensively reviewed by several authors (7, 17, 88, 99). The current article therefore focuses specifically on the endothelial oxidase, the detailed mechanisms of regulation of which are discussed in REGULATED ACTIVATION OF NADPH OXIDASE.

Interactions among different ROS sources and ROS-dependent regulation. Whereas numerous studies have focused on the importance of individual enzymatic sources of ROS generation, it is increasingly clear that there are in fact complex interactions among different ROS sources such that in many pathological settings multiple sources may be involved (Fig. 1). We consider several examples of such interactions.

Mitochondria, in addition to generating ROS, are themselves susceptible to oxidant damage, which can decrease respiratory enzyme activities and mitochondrial membrane potential and lead to greater ROS production (92). Indeed, the term ROS-induced ROS release was coined by Sollott and colleagues (179) to describe the phenomenon during induction of the mitochondrial permeability transition in cardiac myocytes. This phenomenon may apply not only to ROS initially produced within mitochondria but also to nonmitochondrial sources of ROS. Second, the oxidative conversion of XDH to XO (114) has already been mentioned as a mechanism for amplification of ROS production, where the initial ROS generation may derive from a separate source. Recently, this was reported to be an important mechanism contributing to endothelial cell O_2^-· production in response to oscillatory shear stress (113). The latter study also suggested that the level of XO was dependent on a functional NADPH oxidase. Third, the oxidative degradation of BH_4 (where again the initial ROS may derive from one of many sources) can serve to amplify ROS generation through NOS uncoupling. This latter phenomenon has been termed amplification via "kindling radicals" (91). Finally, it has also been suggested recently that mitochondrial ROS generation can lead to NADPH oxidase activation in endothelial cells (136).

A different type of interaction involves the ROS-dependent regulation of the activity of ROS-generating enzymes, i.e., feedback or feedforward regulation. For instance, Rac1-dependent endothelial NADPH oxidase activation and subsequent O_2^-· production mediates a feedback loop leading to increased proteosomal degradation of Rac1 (81). ROS-dependent downregulation of CYP2C has already been mentioned above. On the other hand, Li et al. (101) reported a feedforward mechanism whereby exogenous exposure of smooth muscle cells or fibroblasts to H_2O_2 caused NADPH oxidase activation and endogenous O_2^-· generation, thereby amplifying the vascular injury process. Self-limiting feedback mechanisms may serve to restrict nonphagocytic NADPH oxidase activity to a low output state, whereas the positive feedforward mechanisms may be more important in pathological settings. These paradigms illustrate how small changes in ROS production may be amplified and/or modulated through interactions among different oxidase systems.

TISSUE-RELATED DIFFERENCES IN ENDOTHELIAL O_2^-· GENERATION

It is increasingly appreciated that there may be significant differences in the properties of endothelial cells of different origins, not only microvascular vs. macrovascular (78) but also tissue-related variations (23). However, potential tissue-related differences in endothelial O_2^-· generation have received very little attention. In principle, differences between tissues may be related either to primary alterations in ROS generation or may be secondary to variations in response to stimuli (e.g., cytokines) that provoke ROS production and/or differences in the production of such stimuli. An example of the first possibility is the finding that NADPH oxidase activity of cultured human microvascular endothelial cells is substantially higher than that of cultured human umbilical vein endothelial cells (HUVEC) studied under similar culture conditions (96). Greater ROS generation by microvascular vs. macrovascular endothelial cells may at least partly account for the greater proliferative capacity of microvascular cells in culture (e.g., 1, 78). As an example of altered responsiveness to ROS-generating stimuli,

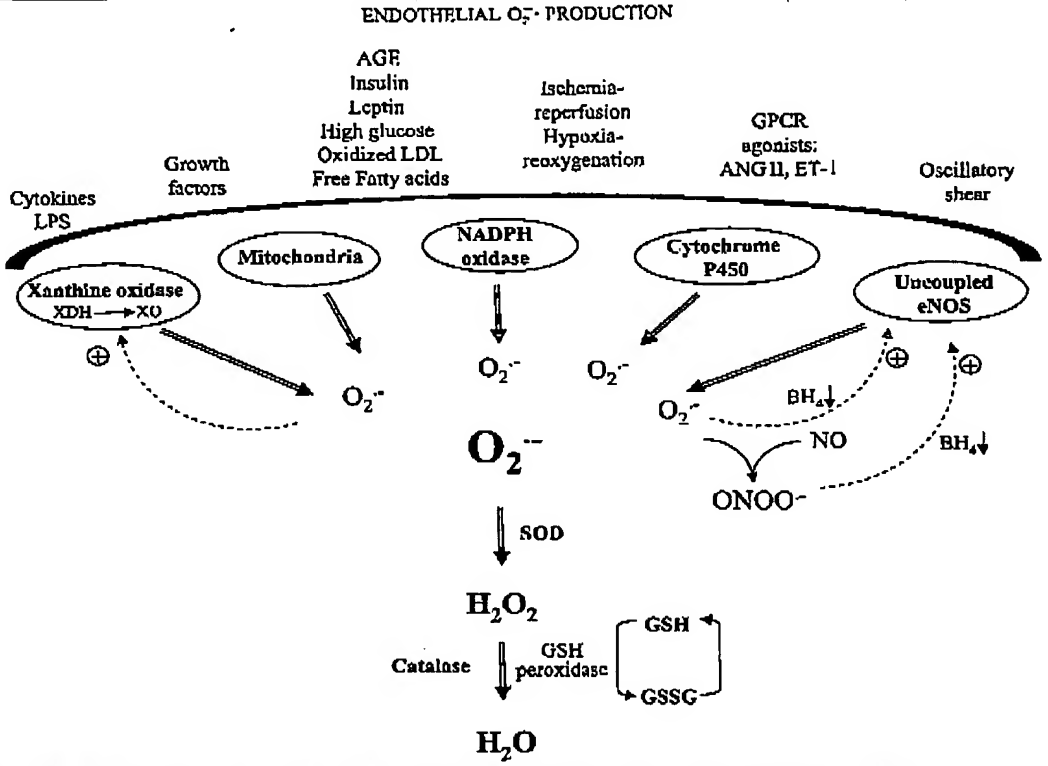

Fig. 1. Schematic diagram showing sources of $O_2^{\cdot-}$ generation in endothelial cells. Potential stimuli for $O_2^{\cdot-}$ generation are shown at the *top*. Dotted lines show feedback effects of $O_2^{\cdot-}$ on xanthine oxidase (XO) or on endothelial nitric oxide synthase (eNOS). AGE, advanced glycation end-product; BH_4, tetrahydrobiopterin; ET-1, endothelin-1; GPCR, G protein-coupled receptor; GSH, glutathione; LPS, lipopolysaccharide; XDH, xanthine dehydrogenase.

it has been shown that the expression of endothelial chemokine receptors (CXCR) and of basal chemokine (e.g., CCL2, CCL5, CXCL10) secretion varies widely according to tissue (63). Similarly, the induction of adhesion molecules on endothelial cells, e.g., in diabetes, can be highly tissue specific (123). More detailed investigation of such differences among endothelia is warranted.

REGULATION OF REDOX HOMEOSTASIS IN ENDOTHELIAL CELLS

The effects of $O_2^{\cdot-}$ and other ROS generated within endothelial cells are critically dependent not only on the amount and sites of production but also on the processes that degrade or scavenge ROS. Several recent reviews have covered the regulation of cellular redox homeostasis (27, 29, 79, 128, 172) and we therefore provide only a relatively brief overview here.

Antioxidant systems influencing redox state comprise nonenzymatic molecules and specific antioxidant enzymes. Nonenzymatic antioxidant molecules in endothelial cells include uric acid, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and glutathione (GSH) (27, 29, 79). The water-soluble vitamin C not only scavenges ROS but also protects vitamin E and GSH against oxidation in cell membranes. Vitamin E is a lipid-soluble, chain-breaking radical scavenger that is considered the most important antioxidant in cell membranes. It may also have nonantioxidant cell signaling functions, e.g., the inhibition of protein kinase C activity (29). GSH is the major low molecular weight thiol antioxidant buffer in endothelial cells (27), serving as a substrate for glutathione peroxidase to eliminate lipid hydroperoxides and H_2O_2, whereby it becomes converted to GSH disulfide (GSSG). Normally, GSSG is maintained at levels <1% of total GSH. The glutaredoxins have functions overlapping with those of thioredoxins (see below) and can reduce GSH mixed protein disulfides.

Important endothelial antioxidant enzymes include SODs, catalase, the thioredoxin system, glutathione peroxidase, and heme oxygenase. The SODs all efficiently convert $O_2^{\cdot-}$ to H_2O_2. The latter is then degraded to water by catalase or glutathione peroxidase. CuZnSOD is suggested to be the predominant SOD isoform in endothelium (18). Knockout of CuZnSOD in mice or inhibition of CuZnSOD results in enhanced vascular $O_2^{\cdot-}$ generation and profound endothelial dysfunction (28, 162). CuZnSOD expression is upregulated by shear stress and is related to cellular redox state (70). Mitochondrial MnSOD expression is also redox sensitive and can be induced by VEGF via the activation of NADPH oxidase (2). The ecSOD isoform is found primarily bound to heparan sulfate on the cell surface and may be especially relevant for regulating extracellular NO bioactivity. In mouse aorta, ecSOD expression was upregulated by angiotensin II (43), whereas on the other hand, mice lacking ecSOD developed significantly higher hypertension in response to angiotensin II infusion than wild-type controls (75).

Thioredoxin reductase together with thioredoxin and NADPH constitutes a ubiquitous oxidoreductase system with antioxidant and redox-sensitive regulatory roles that is abundantly expressed in endothelial cells (172). Thioredoxins efficiently reduce disulfides in proteins, peptides, and GSSG, as well as directly lowering ROS levels through their conserved -Cys-Gly-Pro-Cys- active site (172). Subsequently, the active site disulfide is itself reduced by thioredoxin reductase and NADPH. Thioredoxin has redox-sensitive signaling functions through several mechanisms: *1*) selective stimulation of DNA-binding of NF-κB by reducing a specific cysteine residue in the p50 subunit (172); *2*) increasing AP-1 binding activity via binding to the nuclear redox protein, redox factor 1 (Ref-1); *3*) through binding to signaling molecules such as the MAPKK kinase ASK1, thereby inhibiting its activity—the oxidation of thioredoxin disrupts this binding and leads to increased ASK1 activity. Another protein that binds thioredoxin is vitamin D_3-upregulated protein 1 (VDUP1), which may act as an endogenous inhibitor of thioredoxin. VDUP1 expression is reduced by biomechanical strain or H_2O_2, thereby increasing thioredoxin activity. Interestingly, thioredoxin expression is induced by oxidant stress (172).

Heme oxygenase has indirect antioxidant effects through the degradation of free heme (derived from many hemoproteins and which has potent pro-oxidant actions) as well as the subsequent generation of biliverdin and bilirubin, which have antioxidant properties (128). The constitutive heme oxygenase isoform, HO-2, is ubiquitously expressed in endothelial cells, whereas HO-1 is induced in response to stimuli such as heme, hypoxia, cytokines, oxidized LDL, angiotensin II, NO, peroxynitrite, and H_2O_2 (128). HO-1 expression in response to oxidative stress is a key manifestation of the induction of endogenous cellular antioxidant defense mechanisms.

Despite extensive experimental evidence for an important role of antioxidant systems in redox homeostasis and the finding that antioxidants such as vitamin C can acutely improve endothelial dysfunction related to oxidative stress (130), a clinically relevant therapeutic benefit of antioxidant supplementation remains to be demonstrated, perhaps suggesting that more specific and focused approaches may be required for therapeutic manipulation of these pathways (73).

REDOX SIGNALING MECHANISMS

The modulation of biological signaling pathways by ROS depends on both the upstream ligand-dependent stimulation of ROS production by different enzymatic sources and the specific interactions of ROS with individual downstream pathways. It is clear that ROS may modulate signaling pathways at multiple levels from membrane receptors and channels to various protein kinases and transcription factors in the nucleus. O_2^-· itself is relatively unstable in aqueous solution and is rapidly dismutated to H_2O_2 either spontaneously or by the action of SOD. Therefore, many O_2^-·-dependent signaling events are thought to be mediated through H_2O_2. On the other hand, NO is one of the few biomolecules that can outcompete SOD for O_2^-·; therefore, in settings where there are sufficiently high concentrations of NO present, O_2^-· reacts rapidly with NO in a near diffusion-limited fashion to form peroxynitrite (80). The latter has widely been considered to be a relatively nonspecific toxic species that can oxidize or nitrate a wide variety of biological targets. However, more recent studies indicate that, in vivo, peroxynitrite may interact directly (rather than via breakdown to ·OH or ·OH-like radicals) with specific biomolecules, notably thiols and metal-containing proteins, to modulate signaling events, especially in nonacidic environments (80, 160).

The precise molecules that are targeted by ROS and the exact biochemical reactions involved remain incompletely understood. A common mechanism involves redox-dependent covalent modification of specific cysteine residues on target proteins (38). In the case of tyrosine phosphatase, reversible oxidation of a cysteine residue leads to enzyme inactivation and a secondary increase in activity of tyrosine kinases (e.g., specific MAPKs). Alternatively, the reversible covalent addition of GSH to cysteine residues (or *S*-glutathiolation) may be involved in activating tyrosine kinases (79). In the case of peroxynitrite, in addition to oxidation of thiol and metal-containing protein centers, the tyrosine nitration of proteins could also be involved in signaling, either by negative interference with tyrosine phosphorylation of enzymes and/or by mimicking phosphorylation (80, 160). Redox signaling mechanisms that involve thioredoxin were discussed in a previous section, whereas the effects of ROS on the transcription factor HIF-1 are discussed in *Oxygen sensing* below. More detailed reviews of redox signaling mechanisms were published recently (29, 38, 71, 80, 82).

Although all the endothelial ROS sources discussed previously may potentially be involved in redox signaling, NADPH oxidases seem to be especially important in that they are the main source whose primary function appears to be to modulate redox signaling (15, 17, 50, 88, 99). In the next section, we therefore focus on the mechanisms of regulation of endothelial NADPH oxidase.

REGULATED ACTIVATION OF NADPH OXIDASE

A major attribute of nonphagocytic NADPH oxidases is that not only are they "constitutively" active but their activity is sensitively influenced by a wide variety of (patho)physiological stimuli. Endothelial NADPH oxidase activity is increased by *1*) mechanical forces such as oscillatory shear stress (67); *2*) hypoxia-reoxygenation (77, 136, 141), flow cessation (111), membrane depolarization (4, 140), nutrient deprivation (106), or ischemia (4, 111); *3*) G protein-coupled receptor agonists such as angiotensin II (98, 107, 117, 176) or ET-1 (32); *4*) phorbol esters, which activate protein kinase C (PKC) (47, 95); *5*) growth factors such as VEGF (157); *6*) cytokines such as TNF-α (42, 95) or IL-1 (52); *7*) increased insulin (76), glucose (69), free fatty acids (34, 69), or advanced glycation end products (AGE) (164); and *8*) oxidized LDL, lysophosphatidylcholine, and hypercholesterolemia (133, 144). Either a rapid posttranslational activation and/or the increased expression of oxidase subunits can be involved in the upregulation of O_2^-· production by NADPH oxidases. Although the altered expression of different oxidase components in response to various stimuli has been described in many studies [e.g., increased Nox2 and Nox4 mRNA expression with oscillatory shear stress but decreased expression with pulsatile flow (67)], the underlying mechanisms of transcriptional regulation remain as yet virtually unexplored. However, recent studies have begun to

shed light on the mechanisms underlying the acute activation of endothelial NADPH oxidase.

Regulation by the p47^{phox} subunit. Phosphorylation of p47^{phox} is known to be important for activation of the neutrophil NADPH oxidase, being required for the translocation of cytosolic subunits (p47^{phox}-p67^{phox}-p40^{phox}) to the membrane-located cytochrome b_{558} (8). Several studies using either tissues from gene-targeted mice lacking p47^{phox} or specific inhibitors have likewise shown a crucial role for p47^{phox} in endothelial NADPH oxidase activation by several agonists (angiotensin II, TNF-α, VEGF) (15, 42, 95, 98) and by chronic oscillatory shear (68). The p47^{phox} subunit is also required for angiotensin II-induced MAPK activation (100), TNF-α-induced JNK activation (53), and redox-mediated gene expression (15). The finding that p47^{phox} is essential for PMA-induced activation suggested a role for PKC (95), which was confirmed for TNF-α-induced activation of NADPH oxidase in lung vascular endothelial cells where the atypical PKC isoform, PKC-ζ, was found to phosphorylate p47^{phox} (42). Recently, we demonstrated that angiotensin II-stimulated endothelial NADPH oxidase activation induces the rapid serine phosphorylation of p47^{phox} (1–15 min), which is paralleled by increased p47^{phox}-p22^{phox} binding (i.e., increased complex formation) and increased O_2^-· generation with similar kinetics (98). These results suggest that while basal (constitutive) NADPH oxidase activity may be explained by the presence of preassembled oxidase complexes, enhancement of activity by agonists such as angiotensin II requires the formation of additional complexes. With respect to TNF-α-induced signaling, it has been reported that p47^{phox} associates with the TNF receptor-associated factor TRAF4, which links to downstream activation of JNK (45, 170). The binding of p47^{phox} to TRAF4 may therefore serve as a means to localize the ROS signal to proteins/enzymes associated with TRAF4. Whether similar protein-protein interactions involving p47^{phox} are used as a strategy for the spatial confinement of ROS-mediated signals generated by other agonists remains to be studied.

Studies from our group that have investigated coronary microvascular EC (CMEC) and aortae isolated from p47$^{phox-/-}$ mice or the effects of acute depletion of p47^{phox} by antisense cDNA transfection suggest a more complex role for p47^{phox} (95, 98, 100). Surprisingly, we found that neither the chronic absence of p47^{phox} in knockout cells nor its acute depletion in wild-type cells resulted in a reduction in basal NADPH-dependent ROS production. On the contrary, basal ROS production was slightly but significantly higher compared with wild-type cells or aortae. Consistent with this finding, p47$^{phox-/-}$ aortic rings had mild endothelial dysfunction and increased basal activation of ERK1/2, which were normalized by exposure to ROS scavengers (100). Nevertheless, the acute oxidase response to PMA, TNF-α, or angiotensin II was abolished in p47$^{phox-/-}$ cells (95, 98), whereas in aortic rings angiotensin II-induced endothelial dysfunction was also abrogated (100). These results suggest that p47^{phox} may have a dual role in regulating endothelial NADPH oxidase activity whereby it inhibits basal, constitutive O_2^-· generation but is nevertheless essential for agonist-induced increases in ROS production. A possible explanation for these effects might be that unphosphorylated p47^{phox} is inhibitory when bound to the cytochrome b_{558} in endothelial cells, whereas phosphorylation leads to oxidase activation.

Regulation by Rac1. The other NADPH oxidase regulatory subunit that has been well studied in endothelial cells is the GTPase Rac1, a member of the Rho (Ras homology) family of small (20–40 kDa) GTP-binding proteins that undergo regulation through GTP binding and hydrolysis (49). Rac activity also requires a carboxy terminal geranylgeranyl moiety, which is added by posttranslational modification (isoprenylation) by geranylgeranyl transferase and is required for localization of Rac to the membrane. Geranylgeranyl groups are derived, like cholesterol, from the mevalonate pathway, and the synthesis of both these products is inhibited by HMG-CoA reductase inhibitors (statins). Thus some of the pleiotropic effects of statins may be mediated through inhibition of Rac translocation. Rac in its GTP-bound state is thought to bind to p67^{phox} and activate NADPH oxidase (49). Rac1 can activate NADPH oxidase in the complete absence of p47^{phox} in cell-free systems, but both are probably required for optimal oxidase activation (48). In endothelial cells, PMA-induced O_2^-· production [which is p47^{phox} dependent (95)] is reportedly inhibited by statins in a mevalonate-dependent manner (161), suggesting that both Rac1 and p47^{phox} are required for this response. Basal NADPH oxidase-dependent ROS production requires Rac1 as it is inhibited by a dominant negative Rac1 mutant (66). Similarly, statin withdrawal after chronic treatment in animals stimulates endothelial O_2^-· generation through Rac1-dependent activation of NADPH oxidase, suggesting that basal NADPH oxidase activity is modulated by statins through altered Rac translocation (159). NADPH oxidase-dependent ROS production stimulated by shear stress is also inhibited by dominant negative Rac1 (173), and Rac1-dependent ROS production mediates shear stress-induced endothelial cell tyrosine phosphorylation (173) and the surface expression of ICAM-1 (156). Rac1 appears important in the oxidase response to hypoxia-reoxygenation or ischemia-reperfusion. Thus Rac1 mediates oxidant production in response to hypoxia-reoxygenation in several cell types including endothelial cells (77). Likewise, in HUVEC, depolarization-induced NADPH oxidase activation (which may be relevant to ischemia) required Rac translocation (140). Rac1 is also required for oxidant-dependent expression of MCP-1 induced by nutrient deprivation (106). Another setting in which Rac1-dependent ROS production is implicated is in endothelial cell growth and survival. VEGF-induced endothelial cell migration and proliferation required Rac1-regulated O_2^-· generation (157), whereas the overexpression of a constitutively active mutant of Rac1 resulted in endothelial cell proliferation (105). In HUVEC, Rac1-dependent O_2^-· production led to protection against TNF-α-induced apoptosis (26).

PHYSIOLOGICAL ROLES OF ENDOTHELIAL O_2^-·

Whereas O_2^-· production is implicated in many pathological processes (see later), an important question is whether ROS generation has any physiological roles. A physiological role for ROS would provide at least a teleological explanation for the widespread occurrence of ROS even in health (Fig. 2). In fact, there are several likely physiological roles for endothelial ROS, all of which involve the use of oxygen species to transmit biological information in some way.

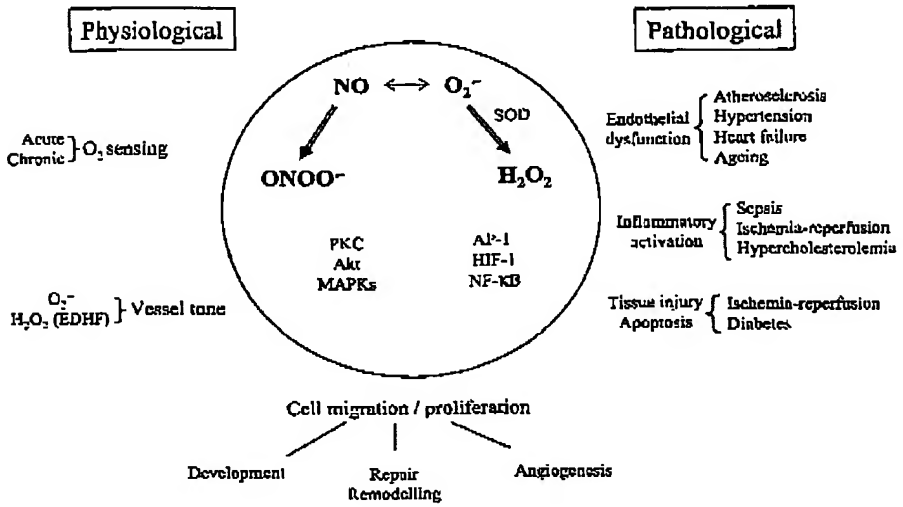

Fig. 2. Potential roles of endothelial reactive oxygen species in physiological and pathophysiological processes (*left* and *right*, respectively). Processes shown at *bottom* may be physiological or pathological depending on the context. Some of the relevant reactive oxygen species as well as potential targets for redox signaling (enzymes involved in signal transduction and transcription factors) are shown in the central circle. EDHF, endothelium-derived hyperpolarizing factor; PKC, protein kinase C; SOD, superoxide dismutase.

Oxygen sensing. Many types of acute and chronic O_2-sensitive processes are involved in cardiorespiratory homeostasis, e.g., an acute increase in ventilation or a chronic increase in erythropoietin production in response to hypoxia (16). Likewise, O_2-sensitive alterations in endothelial function are essential for vascular homeostasis. For example, in the coronary circulation, a modest decrease in arterial P_{O_2} evokes a rapid increase in endothelial production of NO and vasodilator prostanoids that serve to increase blood flow and thus O_2 supply (125). Chronic hypoxia also evokes several adaptive changes in endothelial gene expression.

A substantial body of evidence implicates ROS-producing proteins in the acute sensing of changes in ambient O_2 concentration in different cell types (16). In the carotid body, an ROS-generating cytochrome similar to the cytochrome b_{558} of NADPH oxidase may be involved in sensing modest hypoxia, whereas in other settings alterations in mitochondrial ROS generation are implicated. It is likely that ROS may be similarly involved in the endothelium.

Chronic changes in cellular function in many tissues, including the endothelium, involve redox-sensitive activation of the transcription factor hypoxia-inducible factor-1 (HIF-1), which may increase the expression of genes involved in angiogenesis, energy metabolism, cell proliferation, and vascular remodeling (16). The major physiological importance of these systems is attested to by the finding, for example, that gene-targeted deficiency of HIF-1 results in embryonic lethality (134). Recent studies have begun to delineate the ways in which ROS may regulate gene transcription through HIF-1 (29, 167). The HIF-1 heterodimer comprises an HIF-1α and an HIF-1β subunit. Whereas the protein level of HIF-1β is not much affected by changes in P_{O_2}, HIF-1α undergoes rapid proteosomal degradation through its prolyl hydroxylation by specific prolyl hydroxylase enzymes. The redox regulation of HIF-1 activity appears to be mediated largely through ROS-dependent changes in HIF-1α stability as well as posttranslational regulation of HIF-1 activity. However, the precise mechanisms through which the latter regulation occurs remain unclear, with evidence for regulation through activation of the phosphatidylinositiol 3-kinase/Akt pathway or through a thiol-sensitive mechanism (167).

Regulation of vascular tone. ROS generation may be important in the physiological regulation of vascular tone in at least two ways, first via interactions with NO and second through the direct effects of H_2O_2. It is well established that endothelium-derived NO undergoes a very rapid reaction with O_2^-· that results in inactivation of NO (18). A fundamental aspect of the regulation of vascular tone and blood flow by NO is its rapid sensitivity to alterations in local stimuli (such as increases in shear stress) and the dependence on appropriate local vasodilator actions to achieve integrated increases and/or redistribution of blood flow among specific vascular beds. The physiological local generation of O_2^-· is quite likely to be important in the spatial restriction of the actions of NO, together with other molecules such as hemoglobin (119). In this regard, it is of interest that increased vascular flow is a potent stimulus for the release of both O_2^-· and NO (89). Local SOD activity (in particular ecSOD) may also play an important role in regulating the NO/O_2^-· balance.

Recent studies indicate that H_2O_2 released from the endothelium (after conversion from O_2^-·) may account for EDHF activity in murine and human mesenteric arteries and human coronary arterioles, where it is involved in flow-induced dilatation (112, 115, 118). Endothelial CuZnSOD plays a pivotal role in converting O_2^-· (generated probably mainly by NO synthase) to H_2O_2, to the extent that it was proposed to act as an EDHF synthase. These studies suggest that H_2O_2 production contributes to the physiological regulation of vascular tone in certain vascular beds.

Other functions. ROS can have potent effects on endothelial cell growth, migration, proliferation, and survival (see later), which have been studied mainly in pathological settings. However, it is quite conceivable that such effects may also serve important physiological functions, e.g., during development or reparative processes. Likewise, the effects of ROS on cell adhesion discussed in the next section may be physiologically

relevant in the context of the microvascular inflammatory response to pathogens.

ENDOTHELIAL CELL ACTIVATION AND INFLAMMATION

Inflammation describes the stereotyped response of vascularized tissues to injury and is a major part of innate immunity as well as being involved in the adaptive immune response. It involves the microvasculature, principally postcapillary venules, which are the main sites of vascular leak and leukocyte extravasation. The recruitment and adhesion of leukocytes to endothelial cells and their subsequent emigration from the blood across the endothelium and into the affected tissue is an early step in inflammation, which requires the regulated expression of cell-surface adhesion molecules and other proteins on one or both types of cell (i.e., cell "activation") (46). Adhesion molecules either tether the two cells together and/or act as signals that induce changes in endothelial cell (and leukocyte) structure and function, e.g., raised intracellular Ca^{2+}, ROS production, cytoskeletal rearrangement. Among the adhesion molecules expressed on activated endothelial cells, ICAM-1, VCAM-1, endothelial leukocyte adhesion molecule-1 (ELAM-1 or E-selectin), and P-selectin (CD62) have been well studied. Very little ICAM-1 is normally expressed on endothelial cells but it is greatly increased by inflammatory stimuli such as lipopolysaccharide and cytokines (interleukin-1, TNF-α, interferon-γ) over a time course of hours (46). P-selectin is rapidly translocated to the cell membrane in activated endothelial cells in minutes, whereas E-selectin is newly synthesized and appears after 4–6 h of activation (46). Oscillatory shear is also a potent stimulus for the surface expression of ICAM-1, VCAM-1, and E-selectin in endothelial cells (20).

Substantial experimental data indicate that ROS are potential regulators of endothelial cell adhesion molecule expression and inflammatory microvascular dysfunction (46), for example during sepsis (129). Cytokines such as TNF-α increase VCAM-1 and chemoattractant protein-1 (MCP-1) expression through a redox-sensitive mechanism involving NF-κB, which is inhibited by antioxidants or the NADPH oxidase inhibitor apocynin (155, 165). The role of NADPH oxidase and O_2^-· in this process was highlighted by a study using adenoviral-mediated overexpression of dominant negative Rac1 and SOD, both of which suppressed TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression, through NF-κB inhibition (22). TNF-α-induced rapid upregulation of endothelial P-selectin was also found to depend on O_2^-· generation from the synergistic effects of NADPH oxidase and XO (149). However, it should be noted that a significant component of the ROS released in response to TNF-α may also emanate from neutrophil NADPH oxidase and then induce ICAM-1 expression in microvascular endothelial cells (35).

Endothelial adhesion molecule expression may also be triggered by hypercholesterolemia, ischemia-reperfusion, AGEs, and the renin-angiotensin system. Induction of VCAM-1 by AGE or angiotensin II involves NADPH oxidase and NF-κB activation (131, 164). Hypercholesterolemia-induced leukocyte-EC adhesion and leukocyte emigration also involve NADPH oxidase. Stokes et al. (144) reported that both these processes were significantly attenuated in $p47^{phox-/-}$ mice, an effect that was attributed to reduced O_2^-· production by both the endothelium and white cells on the basis of studies using bone marrow chimeras. Likewise, P-selectin-dependent adhesion of platelets and leukocytes in the cerebral microcirculation was blunted in hypercholesterolemic $gp91^{phox-/-}$ mice (72). In the context of microvascular dysfunction induced by ischemia-reperfusion, XO-derived ROS appear to contribute to an increase in endothelial permeability (114).

ENDOTHELIAL CELL GROWTH, MIGRATION, AND APOPTOSIS

Endothelial cell proliferation, migration, and organization into tubular network structures are critical steps in angiogenesis. Endothelial cell growth and survival are dependent on several factors, including the presence of specific growth factors and cellular interactions with the extracellular matrix. In vitro, growth factor deprivation leads to apoptosis, whereas cell detachment leads to a special form of programmed cell death called anoikis.

Recent studies indicate that low-level regulated generation of ROS is necessary for the processes involved in both angiogenesis and endothelial cell survival. Thus several growth factor receptors are coupled to intracellular production of O_2^-· and H_2O_2 (1, 157). For example, VEGF-induced endothelial cell proliferation and migration was shown to be dependent on O_2^-· generation from NADPH oxidase, in that the mitogenic and chemotactic effects of VEGF were abrogated by three structurally unrelated NADPH oxidase inhibitors (1). Ushio-Fukai et al. (157) showed more specifically that VEGF-induced endothelial cell proliferation, migration, and angiogenesis were inhibited by dominant negative Rac1 or antisense $gp91^{phox}$ oligonucleotides, which reduced VEGF-induced O_2^-· production. Furthermore, in this study, VEGF-induced angiogenesis in an in vivo sponge implant assay was significantly attenuated in $gp91^{phox-/-}$ mice. Other stimuli that induce endothelial cell migration and/or proliferation, such as OxLDL or angiotensin II, also appear to signal these effects via NADPH oxidase-derived ROS (133). Endothelial proliferation induced by shear stress and coronary collateral vessel development are also ROS dependent (51). Hypoxia induces endothelial cell proliferation independent of paracrine effectors, and recently this was shown to be redox sensitive and to involve ROS production by both mitochondria and NADPH oxidase (136). In a perhaps more pathophysiologically relevant model, ischemia-induced neovascularization in the mouse hindlimb was found to be significantly inhibited in $gp91^{phox-/-}$ mice compared with wild type (61).

The migratory behavior of endothelial cells either after injury or during angiogenesis requires significant reorganization of the actin cytoskeleton, a process that appears to require O_2^-· generation. In an endothelial monolayer-wounding assay, ROS production in response to the loss of endothelial confluence was required for the actin cytoskeleton reorganization necessary for endothelial migration and regeneration (116). Likewise, Rac1 is reported to be required for shear stress-induced endothelial cell polarization, which is an important component of the migratory response (168). In the context of hypoxia-reoxygenation, it was reported that the reoxygenation of previously hypoxic endothelial cells induced a burst of O_2^-· that was necessary for translocation of actin filaments to the submembranous network and cytoskeletal reorganization (25).

This phenomenon was suggested to be of relevance in priming endothelial cells for angiogenesis (25).

Apoptosis (programmed cell death) may be considered as a mechanism that counterbalances the effects of cell proliferation. An increase in intracellular ROS production is often observed in apoptotic processes triggered by various stimuli. O_2^-· generated from NADPH oxidase may play a dual role in influencing both endothelial survival and death. For example, Rac1-dependent ROS generation appears to protect endothelial cells against TNF-α-induced apoptosis (26). On the other hand, oxidized LDL or high glucose-induced NADPH oxidase activation promoted endothelial cell apoptosis (44). Likewise, anoikis resulting from detachment of endothelial cells from the extracellular matrix involved a significant rise in the intracellular ROS level (93). The mechanisms by which endothelial cells die or survive under oxidant stress remain unclear, although the downstream activation of JNK is implicated in H_2O_2 and other stress-induced apoptosis, whereas ERK activation is implicated in VEGF-induced endothelial cell survival (71). Mitochondrial-derived ROS play a central role in endothelial cell apoptosis (19, 145). The loss of cytochrome *c* into the cytoplasm and opening of the mitochondrial permeability transition pore are important events in the apoptotic cascade. Loss of cytochrome *c* leads to increased ROS generation, which may activate the mitochondrial permeability transition. Interestingly, the deficiency of mitochondrial cytochrome-*c* oxidase has recently also been linked causally to increased O_2^-· generation during endothelial senescence (169).

INVOLVEMENT OF INCREASED ENDOTHELIAL O_2^-· GENERATION IN CARDIOVASCULAR DISEASE

Endothelial dysfunction. Chronic dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular disorders including atherosclerosis, hypertension, diabetic vasculopathy, and heart failure (18, 57). Whereas endothelial dysfunction encompasses a broad range of abnormalities, a reduced bioavailability of endothelium-derived NO is the most widely studied aspect. A reduction in NO bioavailability in the vessel wall impairs endothelium-dependent vasorelaxation and reduces other beneficial effects of NO such as its inhibition of platelet and leukocyte adhesion and its antiproliferative effects (57, 138). A decline in NO bioavailability may be caused by *1*) reduced expression of eNOS; *2*) deficiency of eNOS substrate (L-arginine) or cofactors (BH_4); and/or *3*) increased inactivation of NO by O_2^-·. The latter is now recognized as a fundamentally important underlying mechanism in most settings (18, 120). It should be noted, however, that the eNOS pathway is subject to regulation by ROS at other levels too. For example, H_2O_2 increases eNOS expression through transcriptional and posttranscriptional mechanisms (30).

In many settings of oxidative stress-related endothelial dysfunction, the increased O_2^-· generation originates not only from the endothelium but also other cell types in the vessel wall, notably vascular smooth muscle cells and adventitial fibroblasts. In the current review, we focus primarily on the role of endothelial O_2^-· rather than these other cellular sources, which have been covered by several excellent reviews (18, 50, 57, 82, 88). The main sources of O_2^-· that are implicated in the genesis of endothelial dysfunction are XO, NADPH oxidase, and uncoupled eNOS. An important point to be reiterated here is that in many cases multiple sources are involved, often as a consequence of ROS-dependent regulation as discussed previously. This is especially relevant in respect of O_2^-· or peroxynitrite-induced degradation of BH_4, which leads to eNOS uncoupling (86, 91), as discussed in the section on *Dysfunctional or uncoupled NOSs*. Good in vivo evidence for a role of ROS derived from uncoupled eNOS in cardiovascular disease models has been relatively limited until recently, with studies in which exogenously administered BH_4 was found beneficial being limited by the fact that BH_4 may have direct antioxidant effects (4b). However, recent studies in which an increase in BH_4 levels driven by the increased gene expression of GTP cyclohydrolase 1 successfully reversed BH_4 deficiency and improved endothelial function (5, 6, 178), now provide more convincing evidence for the in vivo relevance of eNOS uncoupling.

Atherosclerosis and coronary artery disease. Traditional risk factors for atherosclerosis such as hypercholesterolemia and heavy smoking affect endothelial function by increasing ROS production, which decreases NO bioavailability and may convert the normal anti-inflammatory phenotype of the microcirculation to a proinflammatory "activated" phenotype. In addition, ROS production contributes to the oxidative modification of LDL, which plays a critical role in atherosclerosis. Several different ROS sources are implicated. In early atherosclerosis in heritable Watanabe hypercholesterolemic rabbits or cholesterol-fed normal rabbits, reduced NO bioavailability was attributed to increased degradation by O_2^-· derived from endothelial NADPH oxidase activation, which was at least partly AT_1-receptor dependent (163). Likewise, diet-induced atherosclerosis in primates was associated with increased vascular O_2^-· generation, which seemed to be at least partly NADPH oxidase dependent (58). In addition to endothelial dysfunction, increased NADPH oxidase-derived O_2^-· production may also influence the development of atherosclerotic lesions. In $p47^{phox-/-}$ mice studied on an $ApoE^{-/-}$ background, it was found that atherosclerotic lesion area was significantly reduced in the descending aorta compared with $p47^{phox+/+}$ mice, although there was no difference in lesion area at the level of the aortic sinus (10). Consistent with these data, studies on diseased human coronary arteries have shown evidence of increased NADPH oxidase subunit expression together with increased in situ O_2^-· generation in the plaque shoulder area (142). Guzik et al. (56) reported that vascular O_2^-· generation in vessels from patients undergoing coronary artery bypass surgery increased as a function of the number of risk factors for coronary artery disease, with the O_2^-· source being NADPH oxidase. On the other hand, in a recent elegant study in patients with coronary artery disease, Spiekermann et al. (143) showed that increased O_2^-· generation leading to endothelial dysfunction derived from both XO and NADPH oxidase. XO-derived O_2^-· generation is also implicated in endothelial dysfunction in heavy smokers (54) and in hypercholesterolemia (18, 124). NOS uncoupling, too, has been implicated in hypercholesterolemia, smoking (59), and atherosclerosis (91). In a recent study, more direct evidence for an involvement of NOS uncoupling in atherosclerosis was provided by the finding that transgenic mice with endothelium-specific overexpression of GTP cyclohydrolase had reduced aortic atherosclerosis compared with wild types when crossed with ApoE knockout mice

(5). Finally, in patients with coronary artery disease, plasma levels of heparin-mobilizable ecSOD were found to be reduced, which may predispose to oxidative stress (87).

Diabetes. Oxidative stress has emerged as a strong pathogenic cofactor in the development of long-term complications of type II diabetes, such as atherosclerosis, nephropathy, and retinopathy. As mentioned previously, we focus here on endothelial ROS generation in diabetes. Endothelial dysfunction attributable to increased O_2^-· generation is a prominent feature of diabetic vascular disease. As with hypercholesterolemia and atherosclerosis per se, multiple ROS sources are undoubtedly involved. Nevertheless, an increasing number of studies suggest an important role for NADPH oxidase-derived O_2^-· and uncoupled NOS (reviewed in 4b; 99). In aortas from streptozotocin-treated rats, the bioavailability of NO was decreased in the face of increased eNOS expression as a result of increased O_2^-· production from both uncoupled eNOS and NADPH oxidase (64). In this study, a ninefold increase in $gp91^{phox}$ expression was found, and it was suggested that this may be driven by PKC (64). In a porcine model of streptozotocin-induced diabetes, coronary artery oxidative stress was also related to increased NADPH oxidase activity, although this occurred mostly in the media and adventitia (177). In diabetic patients undergoing coronary artery bypass surgery, it was found that NADPH oxidase subunit expression and activity as well as uncoupled NOS-dependent O_2^-· production were significantly higher than in nondiabetics, independent of hypercholesterolemia and at least partly driven by PKC (55, 56). Recently, it has been shown that a key driver of endothelial dysfunction may be the increased oxidation of BH_4, which results in NOS uncoupling. For example, in insulin-resistant rats, oral administration of BH_4 was able to reduce oxidative stress and prevent endothelial dysfunction in the aorta (139). More definitively, in streptozotocin-treated mice, endothelial dysfunction was shown to be improved by the endothelium-specific transgenic overexpression of GTP cyclohydrolase I (6).

Several pathogenic features of type 2 diabetes may be involved in increasing NADPH oxidase activity, which would then promote eNOS uncoupling. These include hyperinsulinemia (76), elevated blood glucose and free fatty acids (34, 69), hypercholesterolemia (133, 144, 148), increased AGEs (164, 177), and increased activation of the renin-angiotensin system (98, 176). Insulin resistance per se may also increase NADPH oxidase activity, at least partly via the renin-angiotensin system (139).

Hypertension. A substantial body of experimental and clinical data implicates increased oxidative stress as being pathophysiologically important in hypertension (18). In patients with renovascular hypertension, excessive oxidative stress was strongly suggested to contribute to impaired endothelium-dependent vasodilatation, which improved after surgery in conjunction with reduced indexes of oxidative stress (62). The link between oxidative stress and hypertension is especially robust with respect to the genesis of endothelial dysfunction, whereas the involvement of O_2^-· in the development of increased blood pressure itself is more contentious. It has been reported that heparin-binding SOD reduced blood pressure in SHR but not in normotensive rats (122) and that antioxidants such as vitamin C and E prevented progression of hypertension in SHR (21). Similarly, in hypertension induced by angiotensin II infusion for up to 7 days, a significant reduction in blood pressure was found with either infusion of liposome-encapsulated SOD in rats (90) or infusion of a peptide inhibitor of NADPH oxidase in mice (132). However, the precise mechanism(s) involved in these antihypertensive effects remain to be established and could include a direct effect on endothelial/vascular function as well as indirect nonvascular pathways. For example, in several studies a dissociation between altered vascular O_2^-· and blood pressure has been found, e.g., in studies in models of low renin hypertension (100b, 141b).

In the last few years, increased NADPH oxidase activity has been reported as a major source of O_2^-· in the vessel wall of experimental hypertension models, including angiotensin II-induced hypertension, renovascular hypertension, genetic hypertension, and DOCA-salt hypertension (86, 100b, 119, 154, 174, 178). However, it should be noted that in many cases this increased activity is found in the vascular smooth muscle and adventitia rather than the endothelium. Studies in hypertensive models associated with activation of the renin-angiotensin system have convincingly shown that increased NADPH oxidase activity (at least in part due to increased subunit expression) contributes both to endothelial dysfunction and elevation of blood pressure per se (21, 50, 85, 90, 100, 132). In the case of low renin hypertension (often studied experimentally using unilateral nephrectomy and administration of DOCA plus salt), an increasing body of evidence implicates ET-1 driven vascular NADPH oxidase activation as being important, especially for endothelial dysfunction (100b, 178). In rats with DOCA-salt hypertension, Li et al. (100b) reported that an ET-1-induced activation of arterial NADPH oxidase, which was ET_A receptor-dependent contributed to endothelial dysfunction in carotid arteries. In this study, in vivo treatment with an ET_A antagonist reduced arterial O_2^-· levels and partially suppressed systolic blood pressure. In studies of DOCA-salt hypertension in mice, Landmesser et al. (86) reported that vascular ROS production involved not only NADPH oxidase but also uncoupled NOS, with the stimulus for eNOS uncoupling suggested to be ROS-induced oxidation of BH_4. In that study, ROS production was inhibited by an NOS inhibitor or in $eNOS^{-/-}$ mice and was also decreased by BH_4 supplementation. However, in rats, Li et al. (100b) did not find evidence for a contribution to O_2^-· generation by uncoupled NOS based on lack of reduction in ROS with NOS inhibitors. The reasons for these differences between studies, apart from the obvious one of species, are unclear. Interestingly, in a subsequent study from the same group, ex vivo gene transfer of GTP cyclohydrolase I into arterial segments was found to increase BH_4 levels, increase basal NO release, and restore endothelium-dependent relaxation, implicating NOS dysfunction as an important contributory mechanism in this model (178). These latter observations may imply that, in some settings, although a reduction in BH_4 levels contributes to eNOS dysfunction this is not necessarily associated with eNOS uncoupling and O_2^-· generation. Therefore, a beneficial effect of BH_4 supplementation is insufficient on its own to prove the existence of eNOS uncoupling; better evidence of the latter process would require more direct data that there is ROS production from eNOS that is related to BH_4 deficiency. In this regard, it is unclear how low BH_4 levels would need to drop in vivo to lead to eNOS uncoupling and O_2^-· generation by the enzyme.

In addition to the studies in low renin hypertension mentioned above (141b, 178), a role for increased endothelial O_2^-· generation by uncoupled eNOS has also been reported in the spontaneously hypertensive rat (24). XO-derived O_2^-· has also been reported to contribute to impaired endothelium-dependent vasodilatation and increased blood pressure in the SHR (146, 122). Finally, antioxidant activities (e.g., SOD and catalase) may be reduced in the SHR.

Heart failure. Increased ROS generation and endothelial dysfunction may play important roles in the development of heart failure. Endothelial dysfunction contributes to the increased peripheral vascular resistance that is a hallmark of congestive heart failure and may be especially important in contributing to reduced exercise tolerance. Indeed, the endothelium has been suggested to be a therapeutic target in this condition (103). A reduced production of NO due to decreased expression of eNOS and a reduction in NO bioavailability due to increased O_2^-· production in the endothelium have emerged as two principal mechanisms that are involved both in experimental and human heart failure (103, 120). In patients with congestive heart failure, both acute and chronic treatment with vitamin C is reported to improve systemic vascular endothelial dysfunction (33, 120). In an experimental model of heart failure induced by myocardial infarction in rats, Bauersachs et al. (11) demonstrated a marked degree of aortic endothelial dysfunction despite an increased expression of eNOS, which was attributable to increased vascular O_2^-· production derived from NADPH oxidase. Our own studies have shown that in experimental pressure overload cardiac hypertrophy and failure, endothelium-dependent (NO dependent) enhancement of left ventricular relaxation is impaired despite unaltered eNOS expression (110) as a consequence of enhanced O_2^-· generation from NADPH oxidase (109). The increased NADPH oxidase activity was subsequently shown to be at least partly due to an increased expression of oxidase subunits (94). Furthermore, impaired endothelium-dependent cardiac function could be restored by treatment with the antioxidants vitamin C or deferoxamine (109). Stimuli that may be important in activating NADPH oxidase in cardiac hypertrophy and heart failure include angiotensin II, ET-1, cytokines, and mechanical forces (60).

A significant body of evidence also supports a role for XO-derived ROS in the systemic vascular endothelial dysfunction seen in chronic heart failure, which can be improved in patients by chronic treatment with the XO inhibitor allopurinol (36).

Ischemia-reperfusion. The increased generation of ROS during reperfusion after ischemia contributes to tissue injury, microvascular dysfunction, increased endothelial permeability, and endothelial "stunning." Tissue injury after reperfusion may have serious consequences depending on the organ, e.g., myocardial infarction or stunning, stroke, and injury after organ transplantation or cardiac bypass surgery. The endothelial generation of O_2^-· plays an important part in these processes. Increased mitochondrial O_2^-· generation on reoxygenation may be involved in increasing endothelial cell permeability (108). Numerous studies support an important role for XO-mediated O_2^-· generation in reperfusion injury. In addition, XO-derived O_2^-· contributes to endothelial dysfunction and activation, which can persist for several days or weeks after reperfusion. Endothelial activation may serve to further enhance ROS production through the recruitment and activation of leukocytes. Increased peroxynitrite formation at reperfusion has also been implicated in cardiac reperfusion injury although the relevance of this in vivo remains to be established (104).

Several studies indicate that endothelial NADPH oxidase may contribute to ROS production during ischemia-reperfusion. Stimuli relevant to ischemia-reperfusion that activate endothelial NADPH oxidase include *1*) hypoxia-reoxygenation (77, 136, 141), *2*) membrane depolarization (4, 140), *3*) flow cessation (111), and *4*) nutrient deprivation (106, 166). In HUVEC, increased ROS generation during reoxygenation after 8 h hypoxia was attributed to XO activation, whereas NADPH oxidase seemed to be downregulated based on studies with gp91ds-tat (141). Hoffmeyer et al. (65) investigated the effects of NADPH oxidase in ischemia-reperfusion injury using $p47^{phox-/-}$ mice subjected to 30 min coronary occlusion and 24 h reperfusion. However, no difference was found in infarct size between wild-type and $p47^{phox-/-}$ mice in this study (65).

Sepsis. Septic shock is characterized by severe hypotension, reduced organ perfusion, loss of vascular responsiveness (hyporeactivity), and in many cases disseminated intravascular coagulation (DIC). An increase in oxidative stress is believed to play a major role in driving or mediating several of these abnormalities, with dysfunction of the endothelium being a major component of pathophysiology. The endothelium represents both a source and a target for ROS released in the vasculature in sepsis, although other cells in the vessel wall as well as inflammatory cells also play important roles. ROS-related endothelial dysfunction in sepsis includes the loss of physiological NO bioactivity; ROS-dependent proinflammatory events such as adhesion molecule expression, recruitment of neutrophils, and cytokine release (see ENDOTHELIAL CELL ACTIVATION AND INFLAMMATION); peroxynitrite production, which may further accentuate these proinflammatory effects and contribute to antioxidant (glutathione) depletion; and the occurrence of DIC, at least in part due to endothelial damage and apoptosis (134b). Many if not all of the main inflammatory pathways implicated in sepsis (cytokines, ET-1, platelet activating factor, etc.) can act on both phagocytes and endothelial cells (as well as other cells) to induce ROS production through NADPH oxidase activation (8, 50), whereas XO-derived ROS is also implicated (114). A major challenge for the future is to assess whether ROS-dependent pathophysiology can be specifically targeted to therapeutic benefit in septic shock.

Nitrate tolerance. Organic nitrates such as glyceryl trinitrate (GTN) are widely used for the symptomatic treatment of ischemic heart disease, where they act by causing venous and arterial dilatation and thereby reducing myocardial work and oxygen consumption. Chronic treatment with nitrates is limited by the development of nitrate tolerance and cross-tolerance to other classes of nitrovasodilators. Although the mechanisms underlying the development of nitrate tolerance are probably multifactorial, studies undertaken by Munzel and colleagues (121, 147) have clearly shown an important role for increased endothelial generation of ROS in this phenomenon. The enhanced endothelial O_2^-· generation has been suggested to emanate both from NADPH oxidase (121) and from mitochondrial ROS, which may act by inhibiting the aldehyde dehydrogenase enzyme (ALDH-2), which is involved in GTN biotransformation in vivo (147).

SUMMARY

The importance of the vascular endothelium in the regulation of cardiovascular homeostasis has become increasingly evident. Endothelial cells have many different functions that are susceptible to modulation by specific local and environmental stimuli. Endothelial activation and dysfunction play a role in the pathogenesis of several cardiovascular diseases. The generation of ROS, in particular O_2^-, by endothelial cells is relevant to their functions both in physiological and pathophysiological settings. ROS exert their effects through several mechanisms including their interactions with NO, their modulation of redox-sensitive signaling cascades, and direct effects on cellular membranes, proteins, and DNA. Several enzymatic sources of ROS are present in endothelial cells, among which a phagocyte-type NADPH oxidase appears particularly important with respect to tightly regulated redox signaling. The effects of ROS generated within endothelial cells are dependent on the amount and site of production as well as the antioxidant balance. Understanding the mechanisms underlying the regulated generation of O_2^- in endothelial cells and the downstream effects of ROS in different pathological settings may help inform therapeutic strategies to tackle endothelial activation and dysfunction in conditions such as hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure.

GRANTS

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